Behavioural, Pharmacological, and Immunohistochemical Investigation of 50 kHz USVs as an Expression of Positive Emotional Arousal in the Long Evans Rat

by
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Abstract

The emission of ultrasonic vocalizations (USVs) in *Rattus norvegicus* is thought to effectively represent an underlying emotional state within the organism manifested at the behavioural level. The main goal of my thesis was to characterize, at multiple levels of analysis, the 50 kHz USVs of the adult rat as an overtly expressed form of positive emotional arousal. In chapter 2, I found evidence of individual differences in 50 kHz emission possibly reflective of a trait that does not merely overlap with approach motivation. The predisposition to emit 50 kHz USVs was found to provide additional information about the USV response to psychostimulant administration beyond approach motivation alone. In chapter 3, I found evidence that various social and non-social behavioural contexts appeared to exert influence on the frequency-modulation characteristics of 50 kHz USVs. My findings suggest that the highest rates of calling and frequency modulation inducible by non-pharmacological stimuli may be observed following exposure of a male rat to a naturally cycling female. Moreover, my research in chapter 3 established that despite context-specific modulation of 50 kHz USVs all such calling could be blocked by antagonism of dopamine receptors. In chapter 4, I utilized microinjections of dopamine into the shell of the nucleus accumbens to establish that dopamine is sufficient to induce 50 kHz USVs. Additionally, my findings from chapter 4 supported the observed association between frequency-modulated 50 kHz USVs and call rate typically induced by psychostimulants. In chapter 5, I used a minimal sensitization protocol with amphetamine to establish that 50 kHz USVs and measures of general ergometric activity could be dissociated. Additionally, in chapter 5 I attempted to find brain region activation patterns associated with calling. My chapter 5 findings failed to

find any direct relations between immunostained brain regions and behavioural expression. However, exploratory analyses suggest possible associations between prefrontal and striatal regions may be involved in the USV behavioural response to amphetamine. In aggregate, my empirical findings are consistent with the existence of a putative subcomponent of the ascending mesolimbic dopamine system responsible for positive emotional arousal reflected by emission of 50 kHz USVs in the rat.

Keywords: 50 kHz USVs; emotional expression; amphetamine; positive emotional states; mesolimbic dopamine.

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AADC	Aromatic amino acid decarboxylase
AcbC	Nucleus accumbens core
AMPH	*D*-amphetamine
ANOVA	Analysis of variance
ARAS	Ascending reticular activating system
BCa	Bias-corrected and accelerated bootstrap
BLA	Basolateral amygdala
CL	Central/caudal linear nucleus
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
DA	Dopamine
DAT	Dopamine reuptake transporter
DMS	Dorsomedial striatum
FM	Frequency-modulated
GABA	Gamma-aminobutyric acid
GBR	GBR-12909
GPCR	G-protein-coupled receptor
HAL	Haloperidol
IEG	Immediate early gene
IF	Interfascicular nucleus
IL	Infralimbic cortex
INJ1	First injection in TIPS

INJ2	Second injection in TIPS
L-DOPA	L-3,4-dihydroxyphenylalanine
MANOVA	Multivariate analysis of variance
M1	Primary motor cortex
MAO	Monoamine oxidase B
MDMA	3,4-Methylenedioxymethamphetamine
mPFC	Medial prefrontal cortex
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NAcSh	Nucleus accumbens shell
NSS	novelty and sensation seeking
OT	Olfactory tubercle
PBP	Parabrachial pigmented area
PBS	Phosphate buffered saline
PL	Prelimbic cortex
PN	Paranigral nucleus
Rac	Raclopride
RL	Rostral linear nucleus of the raphe
RMTg	Rostromedial tegmental nucleus
RRF	Retrorubral field
SAL	Saline
SEM	Standard error of the mean
SN	Substantia niga

SNc	Substantia nigra pars compacta
TIPS	Two-injection protocol of sensitization
TH	Tyrosine hydroxylase
USV	Ultrasonic vocalization
Veh	Vehicle
VMAT-2	Vesicular monoamine transporter-2
VTA	Ventral tegmental area
VTT	Ventral tegmental tail
Zif	Zinc finger-containing transcription factor 268

Chapter 1: General Introduction

Sections ‘The ascending mesolimbic dopamine system,’ ‘The midbrain dopamine complex,’ and ‘Functional heterogeneity of the midbrain dopamine complex’ are adapted from the book chapter:

Brudzynski, S. M., Silkstone, M. J., & Mulvihill, K. G. (2018). Ascending activating systems of the brain for emotional arousal. In: Brudzynski, S.M., (Ed.), *Handbook of Ultrasonic Vocalization. A Window into the Emotional Brain.* Academic Press/ Elsevier: Amsterdam, pp. 239-251.

Additional components of this chapter (descriptions of dopamine and amphetamine) have been adapted from the review paper:

Mulvihill, K. G. (2019). Presynaptic regulation of dopamine release: Role of the DAT and VMAT2 transporters. *Neurochemistry International*, *122*, 94-105.

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Ultrasonic vocalizations in the rodent

A given species-specific behaviour reflects a summation of underlying processes operating within the organism in relation to the context. The overt manifestation of the behaviour itself thus presents an invaluable opportunity for detection and investigation into these underlying processes. The mammalian brain has evolved to support a complex behavioural repertoire capable of fulfilling a wide range of social demands, including, communication (MacLean, 1985, 1988; Newman, 2010). Vocal communication, such as the call emitted by an offspring to engage maternal assistance, has been postulated to have had a central role in the evolution of the mammalian brain (MacLean, 1982, 1985, 1988). The involvement of vocal folds to produce audible signals within a sender and receiver's interaction (i.e., a vocal communicative system; Endler, 1993) has been associated with the development of sociality across a range of mammalian species (Snowdon, 1997; Tyack, 2008). This relation of social behaviour and vocalizations is evident in primates (McComb & Semples, 2005) and also rodents (Eisenberg, 1967; Brudzynski, 2014).

With more than 2,280 species organized into 481 different genera, the order Rodentia represents a considerable proportion of species diversity within the taxonomic class of Mammalia (Wilson & Reeder, 2005; Fabre, Hautier, Dimitrov, & Douzery, 2012). It has been estimated that the proportion of mammals that are rodents may be almost half of all current species. Arguably rodents have thus been immensely successful, which is further supported by the wide range of environments they have been found capable of thriving in, including virtually all terrestrial environments (Catzeflis, Aguilar, & Jaeger, 1992; Fabre et al., 2012). Among many species of rodents, vocal

communication is effectively utilized to fulfill a variety of behavioural roles associated with survival (Okanoya & Screven, 2018). These behavioural roles may include affiliative processes among conspecifics that promote social bonds and reproduction or alternatively a defensive/alarming function necessary for predator detection and escape (Snowdon, 1997; Litvin, Blanchard, & Blanchard, 2010; Brudzynski, 2014).

Although not unique to rodents, a prominent feature that has evolved in the utilization of vocal communication towards survival roles is the elevation of sound frequency employed. Rodents are capable of emitting vocalizations in the ultrasonic range (sound frequencies > 20 kHz, which corresponds to beyond human hearing range). Adult rodents use this sound frequency range as their primary means of social vocalizing in a wide variety of behavioural contexts (Nyby & Whitney, 1978; Brudzynski, 2005, 2009, 2013; Constantini & D'Amato, 2006; Portfors, 2007; Okanoya & Screven, 2018). Subject to extensive predation (Jędrzejewski, & Jędrzejewska, 1993), the use of ultrasound may have evolved in rodents as a form of communication that reduces detection by predators (Arch & Narins, 2008; Brudzynski, 2014). This adaptive advantage results from some predator's inability to detect sound in the ultrasonic range (i.e., birds of prey) but also from the unique physical properties of high-frequency sound (Brudzynski & Fletcher, 2010). The transmission characteristics derived from these physical properties are greater directional control, greater attenuation over distance, and more scattering and reflection by even small objects (Kinsler, Frey, & Adams, 1963; Sales & Pye, 1974). These transmission characteristics make this an ideal communication form to use in the environments many rodents operate within (i.e., underground burrows or forest underbrush; Arch & Narins, 2008; Brudzynski, 2014).

Ultrasonic vocalizations (USVs) have been measured for a wide variety of rodent species (at least 50 species in 30 genera; Sales, 2010). These genera include *Mus* (Liu, Miller, Merzenich, & Schreiner, 2003; Portfors, 2007), *Gerbillus* (Dempster & Perrin, 1991; Dempster, 2018), *Microtus* (Kapusta, Sales, Czuchnowski, 2007; Kapusta & Sales, 2009), *Clethrionomys* (Marchlewska-Koj, 2000), and *Rattus* (Sales, 1972; Barfield, Auerbach, Geyer, & McIntosh, 1979; Kaltwasser, 1990; Brudzynski, 2005). Of particular importance for the current focus is the species *Rattus norvegicus* as it has been the subject of some of the most extensive and systematic investigation of rodent ultrasonic vocalizations.

Ultrasonic vocalizations in the rat

The production of USVs in *R. norvegicus* (henceforth referred to as rat) is accomplished by the formation of a tight opening in the larynx, through which air can be forcefully ejected (Roberts, 1975a, 1975b, 1975c; Johnson, Ciucci, Russell, Hammer, & Connor, 2010; Riede, 2011; Kelm-Nelson, Lenell, Johnson, & Ciucci, 2018). The voluntary contraction of laryngeal muscles that form this tight orifice enables the movement of air to produce a whistle capable of reaching possible fundamental sound frequencies in excess of 100 kHz (Roberts, 1975c; Riede, 2011). Changes in activity of the muscles within the larynx allows for modulation of sound frequency, which is observed as a variety of call types emitted by the rat (Riede, 2013).

The USVs emitted by the rat may be categorized according to their sonographic characteristics (peak frequency and duration of call, sonographic shape, occupied bandwidth, etc.) and the behavioural context in which they are observed (in general, either aversive or appetitive contexts; Knutson, Burgdorf, & Panksepp, 2002;

Brudzynski, 2009, 2013; Simola & Granon, 2018). The two primary types of USVs recognized in the adult rat based on this method of categorization are the ’22 kHz’ and ’50 kHz’ calls. These categories are so termed because of their predominant peak frequency (the sound frequency of maximum power). The 22 kHz USVs typically occupy a sound frequency around 22 kHz ± 3 kHz but could reach 32 kHz and the 50 kHz USVs typically occupy a sound frequency around 50 kHz ± 3 kHz but could reach 60 kHz (Brudzynski, 2005).

The 22 kHz USVs of the adult rat

The 22 kHz calls of the adult rat have been extensively investigated in both sonographic profile (Blanchard, Agullana, McGee, Weiss, & Blanchard, 1992; Brudzynski, Bihari, Ociepa, & Fu, 1993; Brudzynski & Holland, 2005; Simola & Brudzynski, 2018) and functional role in behavioural contexts (Blanchard, Blanchard, Agullana, & Weiss, 1991; Jelen, Soltysik, & Zagrodzka, 2003; Litvin, Blanchard, & Blanchard, 2007). This USV type is characteristically long in duration, although, subgrouping according to duration postulates the existence of long (typically from 300 ms to more than 3000 ms in duration) and short (typically from 20 ms to 300 ms in duration) call subtypes (Brudzynski et al., 1993; Brudzynski, 2015; Simola & Brudzynski, 2018). Both 22 kHz subtypes have a sound frequency maintained between 20 and 32 kHz with a narrow bandwidth (~ 3 kHz) and virtually no frequency modulation (Brudzynski & Holland, 2005).

The emission of 22 kHz USVs has been observed in a variety of aversive situations and is thought to fulfill a higher order defensive function in a colony setting (Blanchard et al., 1991, 1992; Litvin et al., 2007). In response to a predator, the emission

of 22 kHz USVs serves to communicate the danger throughout the colony (Blanchard, Blanchard, Rodgers, & Weiss, 1990; Blanchard et al., 1991). These 'alarm cries' may also be precipitated by generally aversive stimuli such as air puffs (Knapp & Pohorecky, 1995; Brudzynski & Holland, 2005) or acoustic-induced startle response (Kaltwasser, 1990, 1991). The emission of 22 kHz USVs is thought to effectively convey the negative valence of the organisms' emotional state as a form of social transmission (Kim, Kim, Covey, & Kim, 2010). 22 kHz USVs have been posited as representing an underlying emotional state akin to anxiety (Jelen et al., 2003; Brudzynski, 2007).

Adult rats appear predisposed to associate 22 kHz calls with aversive contexts, as seen by a greater amount of freezing behaviour observed in experimentally naïve animals to sound stimuli around the frequency of 22 kHz (Endres, Widmann, & Fendt, 2007). Moreover, learned associations between 22 kHz USVs and aversive situations appear more readily acquired and relatively more resistant to extinction compared with other ultrasonic stimuli (Endres et al., 2007).

The reception of 22 kHz calls is associated with alteration of a variety of general behavioural measures in the receiver (Brudzynski & Chiu, 1995; Commissaris et al., 2000). Reception of 22 kHz ultrasound induces freezing behaviour in a manner comparable to foot-shocks (Nobre & Brandão, 2004) and enhances the acoustic startle reflex of the animal (Inagaki & Ushida, 2017). However, the behavioural patterns observed are not necessarily consistent across playback studies (Schwarting, Kisko, & Wöhr, 2018). In addition to behavioural changes, the reception of 22 kHz ultrasound alters brain activity in several areas including the amygdala, periaqueductal gray, hypothalamus, and thalamus (Beckett, Duxon, Aspley, & Marsden, 1997; Sadananda,

Wöhr, & Schwarting, 2008; Parsana, Li, & Brown, 2012). Thus, the emission of 22 kHz USVs is intimately linked with an adaptive emotional state, the communication of which fulfills vital functions within a species-specific social context. In addition, the emission of 22 kHz USVs serves to express the underlying negative emotional state of the rat. Alternatively, positive or appetitive emotional states of the rat may be expressed by the emission of the other primary type of rat vocalization: 50 kHz USVs.

The 50 kHz USVs of the adult rat

The sonographic characteristics of rat 50 kHz USVs are thoroughly distinguishable from the 22 kHz call type. Broadly speaking, the 50 kHz call type is recognized to possess variability across several acoustic features although predominantly these calls are short in duration (often between 30 and 40 ms) with peak frequencies within 45-55 kHz (Brudzynski, 2005, 2015; Wright, Gourdon, & Clarke, 2010; Simola & Brudzynski, 2018). In contrast to the 22 kHz call type there is extensive frequency-modulation apparent among some 50 kHz USVs (Schwarting, Jegan, & Wöhr; Wright et al., 2010; Burgdorf, Panksepp, & Moskal, 2011; Wöhr & Schwarting, 2013). Thus, 50 kHz USVs may be subdivided into two primary subtypes: frequency-modulated (FM) and constant frequency (flat). Moreover, among the FM 50 kHz calls, there have been a wide variety of call subtypes proposed based on reliable findings of sonographic architecture (Wright et al., 2010; Brudzynski, 2015). However, there is no clear consensus on classification schemes for subdividing the variety of FM 50 kHz USVs observed. Wright and colleagues (2010) enumerated 14 different subtypes based on spectrographic appearance, although, many researchers have used more conservative groupings (Burgdorf et al., 2011; Brudzynski, 2015; Kisko, Himmler, Himmler, Euston, & Pellis,

2015). The general division of 50 kHz USVs into FM and flat subtypes has been used extensively across a range of experimental work (Burgdorf et al., 2008, 2009; Taracha et al., 2012; Inagaki, Kuwahara, Tsubone, & Mori, 2013; Wright, Dobosiewicz, & Clarke, 2013; Taylor, Urbano, & Cooper, 2017).

There is extensive intra-individual stability and pronounced inter-individual differences observed in the emission of 50 kHz USVs (Schwarting et al., 2007; Wright et al., 2010; Taracha et al., 2012; Brenes & Schwarting, 2015). The concept of a 'call profile' has thus been proposed to reflect the unique individual aspects of 50 kHz USV emission in regards to qualitative sonographic parameters such as proportion of subtypes (Wright et al., 2010). The extent to which an individual rats' call profile is altered, depending on the context and stimuli used to induce 50 kHz emission, remains to be fully resolved. Expectedly, given that emission of 50 kHz USVs is a complex behaviour, it may be affected by even subtle aspects of the testing environment such as the use of bedding in the testing chamber (Natusch & Schwarting, 2010).

The emission of 50 kHz USVs has long been established to occur predominantly in emotionally positive contexts with most empirical evidence derived from appetitive scenarios specifically (Barfield et al., 1979; Bialy, Rydz, & Kaczmarek, 2000; Panksepp & Burgdorf, 2000; Simola & Granon, 2018; Wöhr, 2018). Emission of 50 kHz USVs represents an intense overt expression of a positive emotional state and as such has even been proposed to represent an evolutionary antecedent of human laughter (Panksepp & Burgdorf, 2000, 2003). Adolescent and adult rats emit a high number of 50 kHz USVs in non-aggressive social situations including interactions with same-sex conspecifics as well as in mating contexts (Barfield et al., 1979; Knutson, Burgdorf, & Panksepp, 1998;

Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003). There is also intense emission of 50 kHz USVs observed in situations involving expected access to desired food (Brenes & Schwarting, 2015; Opiol, Pavlovski, Michalik, & Mistlberger, 2015), heterospecific play (Panksepp & Burgdorf, 2000), and rewarding forms of brain stimulation (Knutson, Burgdorf, & Panksepp, 1999; Burgdorf, Knutson, & Panksepp, 2000).

The pro-social nature of rat 50 kHz USVs has been well established by studies employing playback of recorded calls or devocalization of the rats (Kisko, Himmler et al., 2015; Kisko, Wöhr, Pellis, & Pellis, 2015; Wöhr, Seffer, & Schwarting, 2016; Schwarting et al., 2018; Wöhr, 2018). The reception of 50 kHz USVs appears to facilitate copulatory behaviour in female rats by increasing receptivity and inducing approach to the male (McIntosh, Barfield, & Geyer, 1978; White & Barfield, 1990). Playback of recorded male 50 kHz USVs to female rats was found capable of eliciting 50 kHz USV emission from females if the male rat is in the vicinity but not directly seen (White, Gonzales, & Barfield, 1993). There is additionally some evidence indicating a greater willingness of females to approach playback of FM 50 kHz USVs in comparison to flat calls recorded from male rats (Snoeren, & Ågmo, 2014). Similar findings have been found for juvenile rats among non-sexual social contexts. Juvenile rats showed preference for conspecifics that vocalized at high rates in comparison to those that called less, and juvenile play has been found to be affected by experimental deafening (Siviy & Panksepp, 1987; Panksepp, Gordon, & Burgdorf, 2002). A role for auditory signaling and 50 kHz USVs as positive signals in juvenile play was further illustrated by devocalization of one or two of the dyads. Rates of play were found to be significantly decreased when

both partners were incapable of emitting 50 kHz USVs (Kisko, Himmler et al., 2015). Moreover, rates of aggressive encounters were significantly greater in pairs of unfamiliar rats when one of them was devocalized and incapable of emitting 50 kHz USVs (Kisko, Euston, & Pellis, 2015).

The playback of recorded adult male 50 kHz USVs may induce approach behaviour and emission of 50 kHz calling in same-sex adult rats (Wöhr & Schwarting, 2007), and additionally, there is evidence that the reception may be associated with phasic dopamine release in the nucleus accumbens (Willuhn et al., 2014). The playback of 50 kHz USVs appears to activate the brain upon reception in a different manner than 22 kHz USVs (Sadanada et al., 2008; Parsana et al., 2012). Burgdorf and colleagues (2008) found evidence that FM calls in particular were associated with positively valenced appetitive behaviours (during mating, play, and aggression) while flat calls were not. Moreover, rats self-administered playback of only FM and not flat 50 kHz USVs (Burgdorf et al., 2008). This finding highlights the possible importance of frequency modulation in reflecting the magnitude of emotional state conveyed by the 50 kHz USVs.

Beyond a specific communicatory role, the emission of 50 kHz USVs occurs in anticipation of social contacts (Knutson et al., 1998; Brudzynski & Pniak, 2002; Willey & Spear, 2012). This anticipatory nature of 50 kHz USVs extends beyond the social domain and may be observed in anticipation of non-social appetitive stimuli such as a food cue (Burgdorf et al., 2000; Willey & Spear, 2013; Buck, Vendruscolo, Koob, & George, 2014) or even exercise (Heyse, Malavar, & Schwarting, 2015). Greater cue-induced anticipatory activity is observed in rats that emit a greater number of 50 kHz USVs even when the associated drive is satiated (Brenes & Schwarting, 2015).

Additionally, expectation of non-natural rewards such as alcohol in dependent rats (Buck, Malavar, George, Koob, & Vendruscolo, 2014), cocaine (Browning et al., 2011; Meyer, Ma, & Robinson, 2012), and amphetamine (Taracha et al., 2014) reliably produces high rates of 50 kHz USV emission. The diverse contexts associated with emission of 50 kHz USVs underlie the idea that their emission, rather than possessing any one singular communicative function, more generally represent the expression of an underlying positive emotional state. The brain system associated with the establishment of such a positive emotional state and the coincident arousal level sufficient for its expression may thus be investigated using 50 kHz USVS in the rat.

The neuropharmacology underlying 50 kHz USV emission

The mammalian brain systems underlying the establishment of emotional states and the associated implementation of relevant behavioural response patterns have been investigated intensely (Olds, 1956; Mogenson, Jones, & Yim, 1980; Rolls, 2000, 2005; Berridge & Kringelbach, 2013). The ground-breaking work of Olds and Milner (1954), which discovered the reinforcing properties of electrical stimulation of certain brain regions in rats, led to substantial progress in understanding positive emotion in the mammalian brain (Olds, 1956; Rolls, 2005; Berridge & Kringelbach, 2013). Elucidation of the pharmacological underpinnings of this positive reinforcement effect and behavioural activation measured by locomotor activity supported a role for the catecholamine neurotransmitter dopamine at some (but not all) brain sites (Rolls, Rolls et al., 1974; Broekkamp, Pijnenburg, Cools, & Van Rossum, 1975; Pijnenburg, Honig, Van der Heyden, & Van Rossum, 1976; Mogenson, Takigawa, Robertson, & Wu, 1979). A similar role for norepinephrine, the other major catecholamine in the central nervous

system, could not be supported (Rolls, Kelly, & Shaw, 1974; Roberts, Zis, & Fibiger, 1975; Clavier, Fibiger, & Phillips, 1976). Central to the role of dopamine in supporting the self-stimulation identified by Olds and Milner (1954) were the ascending dopaminergic projections from the midbrain to forebrain regions associated with limbic input and motor activation (Mogenson et al., 1979, 1980; Rolls, 2005).

These dopaminergic projections have since been implicated in a range of behavioural roles with diverse evidence of the transmitter's function emerging from a variety of empirical approaches (for reviews see: Wise, 2004; Bjorklund & Dunnett, 2007a, 2007b; Iversen & Iversen, 2007; Salamone, Correa, Farrar, & Mingote, 2007; Berridge, 2012; Salamone & Correa, 2012). These projections have been collectively grouped and referred to as the ascending mesolimbic dopamine system (Ungerstedt, 1971; Ikemoto, 2007). Models articulating the function of these ascending mesolimbic dopamine projections have included stimulus-reward learning (Schultz 1997, 1998, 2002, 2007; Ungless, 2004), behavioural activation (Neill & Herndon, 1978; Niv, Daw, Joel, & Dayan, 2007; Robbins & Everitt, 2007; Salamone et al., 2007), motivational state (Wise, 2004; Bromberg-Martin, Matsumoto, & Hikosaka, 2010; Aarts, van Holstein, & Cools, 2011), and incentive salience (Berridge, 2007, 2012; Smith, Berridge, & Aldridge, 2011).

Positive emotional arousal

One function (not exclusive to others) that appears subserved by ascending dopaminergic projections in the mammalian brain is positively valenced emotional arousal (Marrocco, Witte, & Davidson, 1994; Berlucchi, 1997; Jones, 2003). Emotional arousal refers to a general alteration of brain functioning that serves to increase the probability of an internal emotional state with its subsequent increased likelihood of

behavioural initiation (Pfaff, Ribeiro, Matthews, & Know, 2008; Jing, Gillette, & Weiss, 2009; Brudzynski, 2013). Positive emotional arousal specifically relates to the direction the information processing is influenced towards during the development of an emotional state, conceptualized as one of two oppositional valences (positive or negative; Brudzynski, 2007; Barker, 2018).

This positive emotion-specific arousal system is one component of the broader ascending reticular activating system (ARAS) originally characterized by Moruzzi and Magoun (1949). The ARAS functions to activate telencephalic and diencephalic brain structures to promote wakefulness and awareness (Lindsley, Schreiner, Knowles, & Magoun, 1950; Magoun, 1952). While often conceptualized as a unidimensional construct, arousal has been parcellated into multiple sub-systems largely in recognition of the heterogeneous underlying neurochemistry (Berlucchi, 1997; Jones, 2003; Jing et al., 2009; Lee & Dan, 2012; Trofimova & Robbins, 2016). An emotional activating division as part of the general arousal function of the ARAS was first proposed by Routtenberg (1968). Such an emotion-specific arousal system was postulated to function by selectively activating limbic brain structures to modify widespread brain activity.

The neuroanatomy of the transmitter systems that ascend from the brainstem reticular formation may provide insight into the mechanisms that underly this selective limbic activation within the mammalian brain. Evidence obtained from lesioning mesencephalic structures associated with such emotional arousal systems in cats has demonstrated that they are dissociable from general wake-maintenance systems (Webster & Jones, 1988; Denoyer, Sallanon, Buda, Kithama, & Jouvet, 1991). The wide variety of such systems originating from the reticular formation indicates there may be multiple

functional components of the ARAS that serve to modulate widespread brain activity (Jones, 2003; Lee & Dan, 2012). Extensive pharmacological and behavioural investigations over decades have indicated that the transmitter system critical in developing positive and appetitive emotional arousal is the ascending mesolimbic dopamine system from the ventral midbrain to primarily ventral striatum (Robbins, 1997; Wise, 2004; Berridge, 2007; Robbins & Everitt, 2007; Kringelbach & Berridge, 2010; Berridge & Kringelbach, 2013). As previously mentioned, the variety of models articulating the function of the ascending dopamine projections are not necessarily mutually exclusive and often relate to complex behaviour that varies depending on context and time-course from stimulus. Additionally, these models vary in their incorporation of cognitive and emotional explanatory elements. A concept of positive emotional arousal subserved by a component of the ascending mesolimbic dopamine system is concordant with many of these elaborations (including motivational state, incentive salience, and behavioural activation).

The ascending mesolimbic dopamine system was discovered to be a critical neural component underlying 50 kHz USV emission (Burgdorf & Panksepp, 2006; Brudzynski, 2015). Emission of 50 kHz USVs occurred in anticipation of and response to electrical brain stimulation applied to parts of the ascending pathway of dopaminergic fibers (medial forebrain bundle; Burgdorf et al., 2000). Additionally, such electrical stimulation failed to produce 50 kHz USVs upon disruption to this mesolimbic system (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007). Activation of this ascending mesolimbic dopamine system at the midbrain level by electrical or optical stimulation has been demonstrated to induce emission of 50 kHz USVs (Scardochio, Trujillo-Pisanty,

Conover, Shizgal, & Clarke, 2015). Furthermore, local application of pharmacological agents that increase the amount of dopamine in terminal regions of this ascending mesolimbic system has been found to strongly induce 50 kHz USV emission, often above the physiological levels (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Thompson, Leonard, & Brudzynski, 2006). Consideration of the neurotransmitter dopamine is thus important in understanding the 50 kHz USVs of the rat.

The neurotransmitter dopamine

Dopamine is one of the catecholamine neurotransmitters and is produced from the precursor tyrosine. This aromatic amino acid is converted by the rate-limiting enzyme tyrosine hydroxylase (TH) into L-3,4-dihydroxyphenylalanine (L-DOPA; Levitt, Spector, Sjoerdsma, & Udenfriend, 1965). L-DOPA is subsequently converted to dopamine via a decarboxylation accomplished by the enzyme aromatic amino acid decarboxylase (AADC; Christenson, Dairman, & Udenfriend, 1972). In dopamine terminals, this represents the final step of the biosynthetic pathway where dopamine molecules may then be sequestered and packaged into secretory vesicles via transport proteins (Liu & Edwards, 1997). The activity of TH largely governs the overall rate of synthesis, and this enzyme is subject to extensive regulation including inhibition originating from released dopamine itself (end-product inhibition; Masserano & Weiner, 1983). It should be noted that there are a variety of regulatory molecular cascades in addition to this end-product inhibition; however, these fall outside of the scope of the current thesis focus (Zigmond, Schwarzschild, & Rittenhouse, 1989; Tekin, Roskoski, Carkaci-Salli, & Vrana, 2014).

Traditionally, the synthesis of dopamine is thought to occur independently from vesicular packaging, whereby it involves an intermediate cytosolic step for the dopamine

molecules before they are loaded into vesicles. However, there are recent lines of evidence that suggest functional protein associations between the dopamine synthesis enzymes (TH and AADC) and formation and packaging of the secretory vesicles (Chen, Wei, Fowler, & Wu, 2003; Tsudzuki & Tsujita, 2004; Cartier et al., 2010). This association suggests that direct synthesis-packaging coupling occurs, which introduces an avenue for largely bypassing end-product inhibition of TH activity from newly synthesized dopamine molecules.

The degradative and signaling termination mechanisms for dopamine transmission are primarily comprised of the enzymes monoamine oxidase B (MAO; located inside the presynaptic terminal), catechol-O-methyltransferase (COMT; located inside the synaptic cleft), and transporter-mediated removal of dopamine from the synaptic cleft (Kopin, 1985; German et al., 2015). The plasma membrane protein responsible for the reuptake of dopamine from the extracellular space (the dopamine transporter; henceforth referred to as DAT) is a critical regulator of dopaminergic neurotransmission in the central nervous system (CNS) of mammals (Schmitz et al., 2003; Torres, Gainetdinov, & Caron, 2003; Sotnikova, Beaulieu, Gainetdinov, & Caron, 2006; Lohr et al., 2017). Though there are degradative enzymes, this transmembrane reuptake transporter is the primary mechanism of dopamine clearance from the extracellular space (Jones et al., 1998; Benoit-Marand, Jaber, & Gonon, 2000; Gonon et al., 2000). Its transport kinetics helps to shape the temporal and spatial dynamics of dopaminergic action on post-synaptic receptors (Schönfuß, Reum, Olshausen, Fischer, & Morgenstern, 2001; Cragg & Rice, 2004). Thus, it has been a longstanding target of intense research.

Amphetamine as a dopaminergic agent

Many pharmacological agents that manipulate dopamine signaling do so by affecting the reuptake function of the DAT (Reith et al., 2015). Many such agents are well known drugs of abuse in humans (such as cocaine, D-amphetamine, MDMA, or methamphetamine; Heikkila, Orlansky, Mytilineou, & Cohen, 1975; Ritz, Lamb, Goldberg, & Kuhar, 1987; Giros, Jaber, Jones, Wightman, & Caron, 1996; Zhu & Reith, 2008). The drug D-amphetamine (henceforth AMPH) is utilized as an indirect agonist of dopamine to induce 50 kHz USV emission prominently throughout the USV literature (Burgdorf, et al., 2001; Thompson et al., 2006; Wright et al., 2010; Taracha et al., 2012, 2014; Engelhardt, Fuchs, Schwarting, & Wöhr).

AMPH is a prototypical DAT-reversing drug and as such acts as a substrate and reverses the transport activity of the protein (Heikkila et al., 1975; Fischer & Cho, 1979). This reversal leads to DAT-mediated efflux of dopamine molecules from the intracellular side of the presynaptic terminal into the extracellular space in a manner independent of vesicle exocytosis (for more exhaustive reviews see, Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007; Sitte & Freissmuth, 2015). AMPH-induced dopamine release requires functioning DAT molecules so that AMPH may be transported into the terminal (this may be blocked by DAT antagonists such as cocaine; Zaczek, Culp, & De Souza, 1991). From within the terminal, AMPH may then initiate a concert of actions which lead to profound dopaminergic efflux through the DAT protein. In brief, these actions include: depletion of synaptic vesicle stores, which increases cytoplasmic concentration of dopamine (Sulzer et al., 1995), reduction of degradative enzyme activity on cytoplasmic dopamine via AMPH inhibition of MAO (Mantle, Tipton, & Garrett, 1976), and increases

in the frequency of channel-like pore formation enabling extensive non-vesicular release of transmitter (Kahlig et al., 2005).

Dopamine receptors

Given that AMPH acts on the DAT to increase the concentration of dopamine in the extracellular space, the drug indirectly increases activation of all subtypes of dopamine receptors (D_1-D_5). The five dopamine receptor subtypes all belong to the rhodopsin-like (class A) family of G-protein-coupled receptors (GPCRs; Schiöth & Fredriksson, 2005; Beaulieu & Gainetdinov, 2011; Alexander et al., 2013). These dopamine receptor proteins are comprised of seven transmembrane domains and, like other monoamine receptors in the mammalian brain, they generally serve to modulate neuronal signaling. This modulation may function to support the fast neurotransmission of transmitters such as glutamate or gamma-aminobutyric acid (GABA; Missale, Nash, Robinson, Jaber, & Caron, 1998; Beaulieu & Gainetdinov, 2011). As such, most dopamine receptors are localized on non-dopaminergic neurons except autoreceptors, which function in negative feedback cycles in dopamine neurotransmission (Yung et al., 1995; Ford, 2014). The five receptor subtypes are divided into two major groups: D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4). These D_1-like and D_2-like receptor groups are characterized with different (and often opposing) second messenger signaling cascades (Surmeier, Ding, Day, Wang, & Shen, 2007). Within brain areas that receive extensive dopaminergic innervation (e.g., the striatum), these receptor types function to differentially respond to fluctuating levels of dopamine and thereby alter the information processing of cortical afferents (Robertson & Jian, 1995; Goto & Grace, 2005; Gerfen & Surmeier, 2011).

The ascending mesolimbic dopamine system

The projection system originating from the mesencephalon and terminating in diencephalic and forebrain areas is an anatomical system which is functionally defined (Alcaro, Huber, & Panksepp, 2007; Ikemoto, 2007). Indeed, this is consistent with the history regarding the ventral tegmental area (VTA) itself as the original distinction between the VTA and the substantia nigra pars compacta (SNc) was largely based on functionally defined limbic connectivity (Nauta, 1956; 1958; 1960; Dahlstrom & Fuxe, 1964; Ikemoto, 2007). Moreover, the naming of the 'meso-limbic dopamine system' was a functional distinction from the nigro-striatal projections of the A9 group to distinguish these different limbic and motor pathways (Ungerstedt, 1971). This study originally defined the mesolimbic dopamine system as the dopaminergic projections from the VTA to the nucleus accumbens (NAc) and the olfactory tubercle (OT), both structures now considered the ventral striatum (Ungerstedt, 1971; Heimer, 1978; Voorn, Vandershuren, Groenewegen, Robbins, & Pennartz, 2004; Ikemoto, 2007). However, given the extensive projections to non-striatal limbic regions arising from dopamine-containing VTA neurons (Swanson, 1982), the concept of the mesolimbic dopamine system has expanded beyond the terminal area of the ventral striatum (Fields, Hjelmstad, Margolis, & Nicola, 2007; Nieh et al., 2013).

The dopamine neurons in the ventromedial portion of the midbrain, known as the ventral tegmental area of Tsai (VTA), have substantial ascending projections to many areas in the mammalian brain (Andén et al., 1966; Swanson, 1982; Oades & Halliday, 1987; German & Manaye, 1993; Del-Fava, Hasue, Ferreira, & Shammah-Lagnado, 2007). The target structures that receive these projections can be found in the

diencephalon, basal forebrain, and the higher forebrain (Alcaro et al., 2007). Although critically heterogeneous in both cytology and function (Margolis, Lock, Hjelmstad, & Fields, 2006; Roeper, 2013; Lammel et al., 2014; Beier et al., 2015), this housing of cell bodies is most importantly known as the A10 group of dopamine neurons according to Dahlström and Fuxe (1964). This is the group of neurons most commonly associated with the mesolimbic dopamine system which has been researched intensively for decades (Oades & Halliday, 1987; Ikemoto, 2007; Yetnikoff et al., 2014). However, it is widely acknowledged that functionally aligned dopamine neurons contribute to this system from both the A9 group (the SNc) and A8 group (the retrorubral field; RRF; Bjorkland & Dunnet, 2007b; Ilango et al., 2014; Yetnikoff et al., 2014). Thus, the reference to a more general midbrain dopamine complex has been proposed to denote the multi-regional input (Zahm et al., 2011).

As noted above, the ascending dopaminergic projections from the midbrain up to subcortical and cortical structures in the brain comprise one component of the various arousal systems found within the mammalian brain (Marrocco et al., 1994; Jones, 2003). These dopaminergic fibers course along the ventral pathway of the ARAS in that they extend through the lateral hypothalamus to the forebrain (bypassing the thalamus). The stimulation of the midbrain dopamine cells promotes a positive arousal state and is readily self-administered and underlies approach and consummatory behaviour (Rolls, Rolls et al., 1974; Mogenson et al., 1979; Alcaro et al., 2007). The operation of the ascending dopamine projections is far from uniform in function, however, with recent investigations delineating possible functional subsystems. The subsystem supporting emotional arousal appears to operate in parallel with other subsystems originating from

the midbrain dopamine complex (Cohen, Haesler, Vong, Lowell, & Uchida, 2012; Roeper, 2013; Lammel et al., 2014).

The midbrain dopamine complex

The term 'midbrain dopamine complex' (Zahm et al., 2011) appropriately reflects the fact that the A8, A9, and A10 groups (which denote groups of dopamine cell bodies only) all may contribute ascending projections associated with limbic functions (Yetnikoff et al., 2014). These groups of dopamine neurons are located in the anatomical regions of RRF, SNc, and VTA. These anatomical regions also contain neurons of other neurochemical character (including predominantly GABA and glutamate, either co-expressed with dopamine or not; Kosaka et al., 1987; Yamaguchi, Sheen, & Morales, 2007; Morales & Root, 2014). Importantly, however, there are distinct and different proportions of neurotransmitters across the midbrain dopamine regions (Nair-Roberts et al., 2008). In the VTA itself, there are estimates of ~65% dopamine neurons, ~30% GABA neurons, and ~5% glutamate neurons (Nair-Roberts et al., 2008; Dobi, Margolis, Wang, Harvey, & Morales, 2010; Nieh et al., 2013). Though the focus of my research is largely centered on dopamine neurons, it is apparent that both GABA and glutamate neurons in these regions may subserve dopamine function via either local or circuit-wide effects (Hur & Zaborszky, 2005; Yamaguchi, Wang, Li, Ng, & Morales, 2011; Cohen et al., 2012; Neih et al., 2013).

The groups of dopamine neurons labelled as the A8, A9, and A10 cell groups have no clear morphological boundaries; however, there are differentiable topographic and functional projections (Swanson, 1982; Fallon, 1988). The A8 and A9 groups extend lateralward from the A10 group beneath and above the medial lemniscus with the A9

more rostrally located, and the A8 group more caudally (Yetnikoff et al., 2014). Importantly, within the VTA, there are a number of cell groups that can be classified as distinct nuclei or zones (Phillipson, 1979; Oades & Halliday, 1987).

The paranigral nucleus (PN) and parabrachial pigmented area (PBP) are the two major zones found in the rat VTA which are rich in dopamine cell bodies (Phillipson, 1979; Halliday & Tork, 1986). The remaining areas, though less agreed upon, are the parafasciculus retroflexus area and the ventral tegmental tail (VTT), which both comprise dopamine cell body-poor areas within the VTA (Ikemoto, 2007; Bourdy & Barrot, 2012). Some authors have argued that this VTT zone is a distinct nucleus from the VTA and they have so termed it the rostromedial tegmental nucleus (RMTg; Jhou, Geisler, Marinelli, Degarmo, & Zahm, 2009; Lavezzi & Zahm, 2011). In addition, there are a number of midline nuclei which are sometimes considered VTA subregions (Oades & Halliday, 1987). In the rat, these are the central (according to Swanson, 1982 or 'caudal' according to Phillipson, 1979) linear nucleus (CL), interfascicular nucleus (IF), and rostral linear nucleus of the raphe (RL). Both the IF and the CL nuclei are relatively dense with TH-positive cell bodies while, conversely, the RL is more sparsely stained for TH in the rat (Phillipson, 1979; Ikemoto, 2007). It should be mentioned that Yetnikoff and colleagues (2014) have also considered extensions of A10 dopamine cell groupings dorsocaudally (into the ventrolateral periaqueductal gray) and rostroventrally (into the supramammillary nucleus) as functionally significant inclusions to the midbrain dopamine complex concept.

Ikemoto (2007) has convincingly argued that the mesolimbic projections from the midbrain dopamine complex (specifically the VTA and its surrounding areas) are

topographically organized along a medial-lateral gradient to ventral striatal targets in the rat. This is consistent with more recent research showing a similar gradient with functionally distinct populations of dopamine neurons in the mouse brain (Lammel et al., 2008; Beier et al., 2015). Thus, in the rodent brain, the dopamine neurons found in the midline nuclei (mainly in the IF and CL) and the PN appear to preferentially project to the ventromedial striatum (medial accumbens shell and medial olfactory tubercle). While the dopamine neurons found in the PBP appear to follow a medial-lateral gradient (with medial PBP projecting to more ventromedial striatum and the lateral PBP more ventrolateral striatum), they preferentially project to ventrolateral striatum (lateral accumbens shell, lateral tubercle, and accumbens core; Ikemoto, 2007). Moreover, this topographical differentiation between PN and PBP projections extends beyond the ventral striatum with substantially more PN dopaminergic neurons projecting to limbic areas such as the medial prefrontal cortex (mPFC), and the basolateral amygdala (BLA) (Lammel et al., 2008). Virtually no dopamine neurons in the PN project to the lateral accumbens shell, while in contrast, a significant portion of the medial aspect of the SNc does. Moreover, beyond cell body location, there is evidence from input preference, intrinsic properties, and electrophysiological function that suggests there are functionally distinct cellular populations residing in the midbrain dopamine complex (Lammel et al., 2008, 2012, 2014; Margolis, Mitchell, Ishikawa, Hjelmstad, & Fields, 2008; Beier et al., 2015).

Functional heterogeneity of the midbrain dopamine complex

After decades of intense research efforts, the intricate and elaborate nature of the midbrain dopamine complex has remained somewhat elusive. This is due in large part to

the well-established depth of heterogeneity in functional and molecular characteristics of this brain region (Ungless & Grace, 2012; Lammel et al., 2014; Walsh & Han, 2014). These research efforts have nonetheless revealed the midbrain dopamine complex to be a region of integration with many functionally dissociable neural circuits operating in parallel (Lammel et al., 2014; Marinelli & McCutcheon, 2014).

From the accumulated evidence regarding the midbrain dopamine complex acquired across a variety of species (ranging from mice to non-human primates), there has emerged support for a functional subsystem which appears functionally involved with the establishment of positive emotional states (Marinelli & McCutcheon, 2014; Walsh & Han, 2014). This component appears located within the midbrain dopamine complex in the medial posterior portion and is characterized by relatively unique anatomical, molecular and electrophysiological features (Matsumoto & Hikosaka, 2009: Lammel et al., 2014; Beier et al., 2015). As mentioned previously, subgroups of dopamine neurons within the midbrain may be differentiated based on their ascending projections. In the mouse brain, Lammel and colleagues (2008) identified ‘mesocorticolimbic’ neurons (those projecting to BLA, mPFC, NAc core and medial shell) and found that they occupy distinct anatomical territory from the so called ‘mesostriatal’ neurons (those projecting to the dorsolateral striatum). Additionally, this grouping extended to morphological differences (mesocorticolimbic neurons possessing smaller cell diameters than lateral NAc shell and dorsal striatum projecting neurons). Thus, the medial-to-lateral anatomical topography delineated in the midbrain dopamine complex via anatomical projections has a critical importance in parsing the functional subsystems.

In both mice and rats, a variety of molecular markers show expression patterns that reinforce the notion of functionally distinct subgroups of dopamine neurons along a medial-to-lateral pattern within the midbrain complex (Li, Qi, Yamaguchi, Wang, & Morales, 2012; Brown, Day, Dayas, & Smith, 2013; Lammel et al., 2014). Quantitative mRNA expression profiling done in mice revealed that the mesocorticolimbic neurons located predominantly in the medial posterior portion of the midbrain dopamine complex have relatively low levels of plasma membrane dopamine transporter (DAT) and vesicular monoamine transporter (VMAT-2) along with reduced or absent somatodendritic autoreceptors (Lammel et al., 2008). Indeed, Li and colleagues (2012) found a medial-to-lateral expression pattern in the rat (within neurons immunolabelled for TH) with low levels of DAT, VMAT-2, and autoreceptor mRNA in the medial portions of the VTA and higher levels in the lateral portions. Similar results have been found between the VTA and the SN regions in adult rats; with lower DAT relative to TH within the VTA compared to the SN (subgregions unspecified; Brown et al., 2013). These findings represent differences in molecular markers of neurotransmission, which potentially reflect the established motor-to-limbic topography within the midbrain dopamine complex (Fallon 1988; Ikemoto, 2007).

Importantly, these various molecular properties, which help to identify the various subpopulations within the dopamine midbrain complex, also help to explain the critical functional characteristics found among these subpopulations (Marinelli & McCutcheon, 2014). The dopamine neurons found projecting to limbic-associated structures (mPFC, medial NAc shell and core, and BLA) have been characterized via whole-cell recordings as possessing a ‘fast-firing’ phenotype (Lammel et al., 2008). They were found to be

capable of discharging at a higher frequency range for longer periods of time compared with those dopamine neurons projecting to lateral NAc shell or to the dorsal striatum (Lammel et al., 2008). Moreover, within this delineated mesocorticolimbic projection system, it was found that the neurons projecting to the medial NAc shell were differentially modulated by rewarding stimuli in comparison to the mPFC projecting neurons (Lammel et al., 2011). A rewarding stimulus (passive administration of cocaine) upregulated a marker of excitatory synaptic transmission while an aversive stimulus only did for mPFC projecting cells (Lammel et al., 2011). Also, the presence and strength of a hyperpolarization-activated current has been demonstrated to exist along a medial-to-lateral gradient within the VTA (Zhang, Placzek, & Dani, 2010) and has aided distinguishing the mPFC projecting subpopulation from the medial NAc shell projecting cells (Lammel et al., 2008). These various neurophysiological characteristics found among subpopulations of the midbrain dopamine neurons likely serve to create functional differences in release patterns in forebrain target structures. The low autoreceptor inhibition, high vesicular packaging of transmitter, and high firing rate of the medial NAc shell projecting neurons may be what allows for larger and longer lasting dopamine release dynamics in response to natural reward in the NAc shell (Cacciapaglia, Saddoris, Wightman, & Carelli, 2012). And these midbrain subpopulation differences likely contribute to the release differences seen between forebrain regions (Garris & Wightman, 1994; Bassareo, De Luca, & Di Chiara, 2002).

The NAc and 50 kHz USVs

Situated prominently within the mesolimbic dopamine system and embedded in complex cortico-subcortical brain networks, neurotransmission within the NAc

specifically has been extensively studied for its integral role in motivated and emotional behaviour (Jackson, Andén, & Dahlström, 1975; Swanson, 1982; Ikemoto & Panksepp, 1999; Kelley, 2004; Ikemoto, 2007; Cooper & Knutson, 2008; Smith et al., 2011). The NAc has been postulated to represent an interface between limbic brain information and motor capacity for action (Mogenson et al., 1980; Groenewegen, Wright, & Beijer, 1996). Stimulation of reward associated sites in the VTA increases dopamine neurotransmission within the NAc (Fiorino, Coury, Fibiger, & Phillips, 1993). Dopamine signaling within the NAc may then modulate the processing of cortical and subcortical input to effectively re-orient the behaviour of the organism (Nicola, Surmeier, & Malenka, 2000; Goto & Grace, 2005; Britt et al., 2012).

Pharmacological activation of dopamine receptors within this brain structure has been well established to strongly induce 50 kHz USVs (Burgdorf et al., 2001; Thompson et al., 2006; Brudzynski, Komadoski, & St. Pierre, 2012). Pharmacological mapping with AMPH has found that within the NAc the greatest production of 50 kHz USVs results from AMPH application in the ventromedial portion of the shell (Thompson et al., 2006).

Purpose/objectives of current research and structure of overall thesis.

Previous research has demonstrated the widespread utility of 50 kHz USVs in the adult rat as a measurement of positive emotional arousal in a wide variety of experimental paradigms. However, the complexity associated with this form of behaviour has left many important questions unanswered about how 50 kHz USVs express emotional arousal. A central postulation underlying my thesis is that there is a functional subcomponent of the ascending mesocorticolimbic dopamine system associated with positive emotional arousal. An overarching aim of my research was to investigate how 50

kHz USVs in the rat offer a relatively unique behavioural measure of the activity of this emotional arousal system. A major goal of my research was to establish that the 50 kHz USVs of the rat are a detectable manifestation of positive emotional arousal. I argue that these calls are a form of species-specific behaviour that is relatively uniquely associated with this brain system for arousal. The function of this putative subcomponent of the ascending mesolimbic dopamine system is to sufficiently alter the general functioning of the organism in such a way that an emotional state is established. Once established, this emotional state, if sufficient in magnitude, exerts system dominance and is expressed overtly. My research sought to investigate and characterize how 50 kHz USVs relate to activation of the organism through a variety of means (both pharmacological and non-pharmacological stimuli) in adult male rats. To accomplish this characterization, the emission of 50 kHz USVs was investigated at the behavioural, pharmacological, and brain network levels across four studies. The studies conducted in chapters 2 and 3 were primarily investigations of 50 kHz USVs at a behavioural level, chapter 4 was primarily an investigation at the pharmacological level, while the study in chapter 5 was intended to be at the brain network level using immunohistochemistry.

In chapter 2, I investigated whether the behavioural expression of 50 kHz calling could be empirically decoupled from other forms of behaviours associated with the general activation of the mesolimbic dopamine system. I focused on individual differences observed among the forms of behavioural expression related to mesolimbic dopamine activity. I then looked at the variance in these forms of behaviour and investigated their predictive utility for 50 kHz calling following pharmacological activation of the animal's brain using AMPH. I was interested in whether the different

aspects of reward (the 'liking' and 'wanting'), which are both related to the functions of the mesocorticolimbic dopamine system, could be separated from 50 kHz USV production for measuring individual differences. For all subjects, I recorded their sucrose preference (measure of 'liking'), their latency to approach and consume a palatable food stimulus (measure of 'wanting'), and 50 kHz USV emission at baseline and after AMPH. AMPH was used in this context to provide an activating stimulus capable of robustly inducing 50 kHz call emission. An individual rats' baseline predisposition to emit 50 kHz USVs was then compared with these other behavioural predictors of response to AMPH (both liking and wanting aspects) to investigate a possible unique association of 50 kHz calling as an individual trait. Additionally, since the use of AMPH to pharmacologically induce 50 kHz calling is generally regarded as the gold standard I hoped to better characterize the nature of 50 kHz USVs in response to AMPH without any prior sensitization. It was generally hypothesized that a measure of predisposition to emit USVs would provide non-overlapping information about the USV response to AMPH when compared with measures of hedonia and approach motivation.

In chapter 3 I investigated the use of several non-pharmacological stimuli for inducing 50 kHz USV emission. I used a between-subjects paradigm to look at the effect of two forms of non-social stimuli (access to the consumables of Fruit Loops or 2% ethanol v/v) or two forms of social stimuli (exposure of naïve rat to a naturally cycling female or reuniting with a same-sex cage partner). For each of these conditions, 50 kHz USV emission was recorded and analyzed across 4 sessions. 50 kHz calls were coded into different subtypes based on sonographic architecture and the parameters of individual calls were calculated. Following these sessions of stimulus-induced 50 kHz

calling, a dopamine receptor antagonist was administered to establish if the non-pharmacologically induced 50 kHz USVs could be pharmacologically blocked. A central aim of this study was to investigate if the subtypes (forms of frequency-modulation) observed among 50 kHz USVs are associated with a specific behavioural context. An additional aim of chapter 3 was to determine the dependency of such context-associated calling on dopamine signaling. Common dependency on dopamine would conform to the notion that the 50 kHz calling induced by varied behavioural contexts are supported by a single underlying neurochemical system.

In chapter 4 I investigated the use of the native transmitter dopamine for inducing 50 kHz calling from direct intracerebral application within the nucleus accumbens of the adult male rat. I also compared the effects of these microinjections of dopamine on both 50 kHz call rate and sonographic characteristics with microinjections of AMPH into the nucleus accumbens. In addition, I compared the effect of antagonism to the dopamine reuptake transporter or dopamine autoreceptor in combination with dopamine microinjections to AMPH. This study sought to determine the capacity of dopamine signaling locally in the nucleus accumbens to alter the behavioural expression of the organism in the form of 50 kHz calling. Moreover, microinjections of dopamine were utilized to characterize the nature of pharmacologically induced 50 kHz USVs using AMPH. I recorded 50 kHz USV emission including the call rate, latency to call, call profile, and sonographic characteristics of individual calls and used these measures to compare them across injection conditions. A central question of interest was whether dopamine-induced 50 kHz USVs would share the same relationship with frequency modulation that is conventionally observed with AMPH-induced 50 kHz calls (Wright et

al., 2010; Taracha et al., 2012). In such a case, an increase in calling is associated with an increase in frequency-modulation of calls, which has been interpreted as suggesting increased behavioural activation and concomitant emotional arousal (Burgdorf et al., 2011). It was hypothesized that dopamine would be found to be sufficient for inducing and increasing frequency-modulation in a manner comparable to AMPH.

In chapter 5, I investigated possible associations among several forebrain regions and AMPH-induced 50 kHz calling. Specifically, I was interested in whether the behaviour of 50 kHz USV emission would be associated with differential patterns of brain activity as indicated by expression of the inducible transcription factor Zif-268 (Zif). I was particularly interested in prefrontal and striatal regions in the forebrain that are located along the midline (the prelimbic and infralimbic mPFC regions and medial portions of the dorsal and ventral striatum). These are regions that most closely correspond to the putative anatomical topography of the mesocorticolimbic dopamine projections putatively associated with the subcomponent for emotional arousal. For the study in chapter 5, I recorded both 50 kHz USV emission and general ergometric activity (measure of general energy of motor activity per time unit) of the animal across several timepoints either preceding or following systemic AMPH application. I sought to investigate the association of brain activity and measured 50 kHz USV emission and used the recorded ergometric activity as a form of positive behavioural control. A central aim of this study was to indicate at a brain region level whether the brain activation induced by AMPH would relate to measured aspects of 50 kHz USV emission.

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Rationale for Chapter 2

The administration of AMPH has been used extensively to induce positive emotional arousal (for review see Rippberger, van Gaalen, Schwarting, & Wohr, 2015). The emission of 50 kHz USVs is one of a variety of behaviours often observed following application of the drug. However, there are inter-individual differences in the USV behavioural response to AMPH often noted. While most rats emit 50 kHz USVs following amphetamine there are a small number that do not show a significant behavioural response, and additionally extensive variance in the magnitude of response is exhibited across a given sample. While these individual differences are often utilized productively in models of drug addiction and sensitization, it is not clear if they are fully accounted for by these models. Understanding the extent to which the variance in USV response following acute administration of AMPH may represent different behavioural predispositions was one general aim of chapter 2.

The motivational and emotional components underlying a given behaviour may in theory be separable in function. This is evidenced at the behavioural level when motivated behaviour occurs in the absence of any associated hedonic response or other overt aspects of a positive emotional state (Barker et al., 2014). A second aim of chapter 2 was to assess whether predisposition to emit 50 kHz USVs provides additional information about the USV behavioural response to AMPH than individual behavioural measures of hedonia and motivational approach. If determined that these operationally defined measures are dissociable it would support the utility of AMPH-induced 50 kHz USVs as a behavioural measure for providing insight on the internal state of the organism. Moreover, such a determination helps to elucidate the functional divisions

argued to be operating within the ascending mesolimbic dopamine system as activated by a drug like AMPH.

Chapter 2: Individual behavioural predictors of amphetamine-induced emission of 50 kHz vocalization in rats

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Introduction

Adult rats emit vocalizations in the ultrasonic frequency range that serve to convey their motivational and emotional state to other conspecifics (Brudzynski, 2009, 2013). This has made the detection and measurement of these USVs a highly useful index of emotionality in a wide variety of experimental settings (Wöhr & Schwarting, 2013). The recorded emission of so-called 50 kHz USVs provides a quantifiable metric of positive emotional states that does not require conditioning (Brudzynski, 2009, 2013). This call type is elicited by appetitive, positive behavioural situations (Burgdorf & Panksepp, 2001; Burgdorf et al., 2008), rewarding brain stimulation (Burgdorf, Knutson, & Panksepp, 2000; Scardochio, Trujillo-Pisanty, Conover, Shizgal, & Clarke, 2015), and administration of psychostimulants, e.g., amphetamines (Mahler et al., 2013; Simola, Frau, Plumitallo, & Morelli, 2014). Due to the unconditioned nature and generalizable characteristics of this form of behavior, recording of 50 kHz USVs has been utilized in many models of drug addiction and sensitization (Panksepp, Knutson, & Burgdorf, 2002; Browning et al., 2011; Maier, Abdalla, Ahrens, Schallert, & Duvauchelle, 2012; Mahler et al., 2013; Simola et al., 2014; Barker, Simmons, & West, 2015). The emission of 50 kHz USVs is generally dependent on the activity of the mesolimbic dopamine system (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007; Scardochio et al., 2015), and associated with increased dopamine activity in the NAc (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Thompson, Leonard, & Brudzynski, 2006; Hori et al., 2013).

50 kHz USVs are categorized and characterized by their respective acoustic parameters, with the degree of sound frequency modulation appearing to be the best index of positive emotional arousal (Brudzynski, 2009, 2013, 2015; Burgdorf, Panksepp,

& Moskal, 2011; Mahler et al., 2013). Due to differing degrees of frequency modulation observed across individual calls, 50 kHz USVs can be subdivided into 'flat' (non-modulated) and frequency-modulated (FM) call subtypes. These call subtypes are characterized by different sonographic profiles though they share the same average peak frequency range around 50-55 kHz (Brudzynski, 2009, 2013, 2015). These call subtypes are associated with distinct behaviours and contexts, with FM calls most strongly related to reward-associated contexts such as psychostimulant administration (Burgdorf et al., 2008; Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009; Takahashi, Kashino, & Hironaka, 2010; Ahrens et al., 2013). It has been suggested that 50 kHz USVs may represent motivational markers whereby they are elicited in association with the expectation/anticipation of a reward (Knutson, Burgdorf, & Panksepp, 1998, 1999; Burgdorf et al., 2000). This is supported by findings of anticipation-related 50 kHz USV emission to psychostimulants which directly stimulate dopamine release in the NAc (Ma, Maier, Ahrens, Schallert, & Duvauchelle, 2010; Browning et al., 2011; Ahrens et al., 2013; Simola & Morelli, 2015). Moreover, beyond drugs that directly stimulate the mesolimbic dopamine system, there is evidence of non-DAergic agents (e.g., morphine and sucrose) having the capacity to elicit anticipatory or conditioned 50 kHz USVs (Browning et al., 2011; Simola et al., 2012, 2014).

Two conceptual components related to an animal's response to a given reward are the hedonic value (the 'liking') and the motivation to consummate (the 'wanting') (Wise, 2004; Berridge & Kringelbach, 2013). In the present experiment, an L-maze apparatus was used. The L-maze removes much of the aspect of reward learning typically associated with T-maze performance (Tolman, Ritchie, & Kalish, 1946). Evidence from

rodents in the T-maze indicates that differences in dopamine levels result primarily in differences in the motivation to obtain reward (Robinson, Sandstrom, Denenberg, & Palmiter, 2005; Robinson, Rainwater, Hnasko, & Palmiter, 2007; Howe, Tierney, Sandberg, Phillips, & Graybiel, 2013). Thus, a measure of latency in the L-maze should index an individual rats' *motivation* to approach and consume a food reward and therefore should strongly positively correlate with individual differences in 50 kHz USV production.

The consumption of sucrose has been used widely as a measure of hedonia in rats (Sclafani & Clyne, 1987; Mateus-Pinheiro et al., 2014) and appears dependent on the function of dopamine in the NAc (Hsiao & Smith, 1995; Hajnal, Smith, & Norgren, 2004). Sucrose solutions, that are readily self-administered and consumed, induce significant 50 kHz USV production though without the same sensitization effect noted with cocaine self-administration (Browning et al., 2011). Positive relationships are found between individual differences in sucrose intake and psychostimulant self-administration (cocaine and AMPH) (Sills & Vaccarino, 1994; DeSousa, Bush, & Vaccarino, 2000; Gosnell, 2000). Moreover, there have been findings of positive relationships between individual differences in the preference for sucrose and high 50 kHz USV emission behavioural phenotype (Mällo et al., 2007; Burgdorf et al., 2009; Mateus-Pinheiro et al., 2014). Following selective breeding based on 50 kHz USV emission, the high-line rats (which more readily emit 50 kHz calls) showed evidence of greater sucrose preference compared with random-line rats (Burgdorf et al., 2009). Thus, a measure of sucrose preference should index a rat's individual *hedonic response* and should positively

correlate with individual differences in 50 kHz USV production, providing some accounting for the 'liking' aspect of reward.

AMPH has been extensively used as a psychostimulant inducer of 50 kHz USVs with strong individual differences in response to systemic administration both in regard to call rate ('low callers' versus 'high callers') as well as subtypes emitted ('call profile') (Wright, Gourdon, & Clarke, 2010; Taracha et al., 2012, 2014; Ahrens et al., 2013). Additionally, Engelhardt, Schwarting, and Wöhr (2018) recently found evidence of a positive relationship between spontaneous and AMPH-induced 50 kHz call emission. Moreover, these inter-individual differences in baseline 50 kHz USV production were potentiated by injections of AMPH and appeared related to trait-like differences in approach behaviour to 50 kHz USV playback. It should also be noted that inter- and intra-individual differences in calling extend beyond AMPH-induction and are found when 50 kHz USVs are elicited via non-pharmacological methods (Schwarting, Jegan, & Wöhr, 2007). High caller rats that produce more 50 kHz USVs after systemic AMPH administration also show conditioned place preference to AMPH and a greater proportion of FM calls after AMPH when compared to low callers or controls (Ahrens et al., 2013; Taracha et al., 2014). In contrast, it has been reported by several researchers that locomotor activity and emission of 50 kHz USVs appear to be dissociable behavioural phenotypes with only partial overlap (Maier et al., 2012; Ahrens et al., 2013; Simola et al., 2014; Taracha et al., 2014; Engelhardt et al., 2018). High caller rats that show behavioural sensitization to repeated AMPH injections in their production of 50 kHz USVs do not necessarily show locomotor sensitization of a comparable nature (Ahrens et al., 2013; Taracha et al., 2014). A similar dissociation between reward related behaviours

is observed between 50 kHz USV emission after AMPH and social play behaviour, whereby the individual behavioural phenotypes for each behaviour are not necessarily positively correlated (Manduca et al., 2014; Garcia & Cain, 2016). These results indicate that multiple subsystems likely underlie an individual animal's orientation to rewards and raise the question of what AMPH-induced 50 kHz USV emission represent.

The present study set out to determine if the aspects of reward (liking and wanting) and USV production (individual predisposition to call) could be dissociated in a non-sensitization model of acute AMPH induction of 50 kHz calling. To date there appears to be a strong convergence of evidence indicating 50 kHz USVs as motivational markers indexing the 'wanting' component of reward (Burgdorf et al., 2000; Ma et al., 2010; Browning et al., 2011; Ahrens et al., 2013). This focus on the motivational aspect of 50 kHz USVs is especially the case in regards to research involving drugs of abuse (Mahler et al., 2013; Simola & Morelli, 2015). However, there are discrepancies in this narrowed notion of 50 kHz USVs as signals of individual motivation phenotype as they are often dissociable from other reward-related behavioural phenotypes (Barker et al., 2015; Garcia & Cain, 2016). Additionally, most research that has recently found a dissociation of AMPH-induced calling behaviour and reward-related behavioural phenotypes has employed repeated administration protocols of sensitization (Taracha et al., 2012, 2014; Simola et al., 2014). To uncover the nature of what AMPH-induced 50 kHz USVs represent in regards to individual characteristics associated with reward and calling, the present study uses a non-sensitized, non-anticipatory AMPH-induction protocol and a within-subjects design. For each animal measures of hedonic drive (sucrose preference), motivation for reward (latency to approach and consume a food

reward in an L-maze), and predisposition to emit 50 kHz USVs (calls after saline) were used to predict call rate after 1.5 mg/kg of systemic AMPH. It was hypothesized that call rate after AMPH would reflect a measure of an individual's motivation to approach reward, their hedonic drive, and also their predisposition to emit 50 kHz USVs. These AMPH-induced 50 kHz USVs thus may represent a general positive emotional state rather than simply motivational drive. Therefore, it was hypothesized that each of these variables would predict USV response after AMPH in a dissociable fashion (see Figure 2-1).

Methods

Subjects

Forty-six male Long Evans rats (Charles River Laboratories, Saint-Constant, QC, Canada) were used for all behavioural procedures. All animals were approximately 53 (±1) days old at the beginning of the study with an average body weight of 288 g (*SD* = 26.1 g, min. = 234 g, max. = 344 g). At the end of the study animals had an average body weight of 390 g (*SD* = 42.1 g, min. = 315 g, max. = 474 g) and were approximately 78 (±1) days old. In accordance with Brock University protocols for laboratory handling, all animals were housed in polycarbonate cages (19" x 10½" x 8") with dust-free beta-chip® bedding (autoclaved wood fibers, Northeastern Products Corp., Warrensburg, NY, USA) and with a plastic tube inside for hiding. The housing room was maintained with controlled room temperature (23° C ± 2° C) and humidity (40-60%) conditions. Subjects were housed in pairs with a maintained 12:12 light/dark cycle and *ad libitum* access to food and water. Animals were gently handled once before the initial behavioural procedure. All research protocols were approved by Brock University Animal Care and

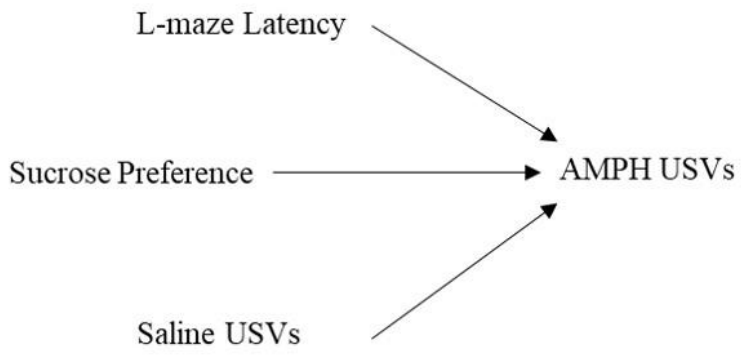

Figure 2-1. Diagrammatic representation of predictive model representing L-maze Latency, Sucrose Preference, and Saline USVs as independent predictors of the response to AMPH measured via USV rate.

Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

Procedure overview

All animals underwent behavioural procedures in the same order, with the procedures separated by 3 days between them. The behavioural procedures involved measuring sucrose preference (using 10% w/v solution versus water) and latency to approach a palatable food cue in seconds (s) before receiving subcutaneous injections followed by recording of USVs on two separate occasions. The first injection and recording was saline (0.2 ml), 3 days later the animals received an injection of AMPH (1.5 mg/kg in 0.2 ml saline s.c.).

Sucrose preference

Sucrose preference was measured across two testing days for all subjects. Prior to testing, all animals were habituated to a 10% sucrose solution (w/v) initially via overnight exposure (Techniplast polycarbonate drinking bottles were left in housing cages at the start of the dark cycle and removed at the beginning of the light cycle). Animals were subsequently habituated to the testing procedure that consisted of placing each cage partner in their own testing cage (with the home cage bedding split between both cage partners). Bottles containing either water or sucrose solution (10% w/v) were then added to the cage for 15 minutes of test time. The weight of the bottles was determined before and after the testing period and the difference was then used to estimate the amount consumed from each bottle (sucrose and water) in grams. Sucrose preference was then determined as the amount of sucrose divided by total amount from both bottles. Following this habituation, all animals were tested the following two days in accordance

with this procedure, with placement of bottles and testing order counter-balanced across the testing days. For all testing days, water restriction was employed by removing water bottles for two hours preceding the test procedure. All testing took place between 10:30 am and 4:30 pm during the light cycle.

Latency to approach and consume reward

The latency to approach and consume a palatable food stimulus, either chocolate chips (semi-sweet chocolate) or Fruit Loops (sweetened, fruit-flavored cereal, Kellogg Company), was measured for all subjects in seconds. Prior to testing, all animals were exposed to their respective food stimulus (chocolate chip or fruit loop) overnight. Additionally, all animals underwent habituation exposure to the L-shaped alley (L-maze) apparatus made of washable white plastic (Technical Services, Brock University). The L-maze apparatus was a modified T-maze with one arm permanently blocked. The length of the alley was 74 cm (width of 12 cm), and the length of the arm was 33 cm (width of 12 cm).

The rat would be placed into the apparatus at one end (the ‘start’ end) opposite of the food stimulus. All rats were placed in at the same end and the location of the food stimulus never changed and was constant across all rat testing. To consume the food stimulus the rat had to travel around a single bend of the alley. During habituation training the subject was given 300 s to approach and consume the food stimulus. If the subject failed to consume it was removed from the apparatus and its non-consumption was recorded. If the subject consumed within 300 s it was placed back in the apparatus with another food stimulus until 300 s total time had elapsed. Subjects underwent habituation exposure until they approached and consumed the food stimulus such that by

the end of habituation training all subjects approached and consumed the food stimulus within 300 s.

Following habituation training, all subjects were tested in the apparatus across five recorded 'runs' with a maximal amount of time allowed of 300 s. Latency to approach and consume the food stimulus was recorded in seconds. This testing was conducted twice for every subject on two consecutive days with order of exposure counter-balanced across sessions (such that no rat was placed into the apparatus in the same order in both testing sessions). Both habituation training and testing took place with the same experimenter, who stood in the same location (behind the start arm) for all sessions. The location of the apparatus did not change throughout the entire duration of the procedure. All testing took place between the hours of 10:00 am and 6:00 pm during the light cycle.

Subcutaneous injections and behavioural recordings

Following the end of the L-maze apparatus testing, all subjects were briefly habituated to the recording procedure. This habituation involved removal of the animal from their housing cage and placement into a holding cage (same dimensions as housing cage) for 10 minutes before placement in a recording chamber (25 cm wide x 18 cm deep x 18 cm height) for 10 minutes following gentle handling to mimic subcutaneous injections. The time spent in the recording chamber employed identical settings (lighting, placement, and experimenter) as was used for the USV recording sessions.

The day following habituation, all subjects were subcutaneously injected with physiological saline (0.9% NaCl in sterile H_20) at a volume of 0.2 ml. After injection, they were placed back into the holding cage for 10 minutes before being placed into the

recording chamber for 5 minutes with any subsequent USV production recorded (for details see section 'USV recording and analyses'). Three days after the saline recording, all subjects were subcutaneously injected with D-amphetamine (at a dose of 1.5 mg/kg in a vehicle of 0.2 ml sterile saline). The D-amphetamine sulfate (Sigma-Aldrich, Great Britain) solution was prepared fresh the day of injections. Animals were placed back into their holding cage immediately following the AMPH injection for 10 minutes before being placed into the recording chamber for the next 10 minutes. Immediately following the end of the recording session, the animal was decapitated, and the brain was extracted for use in other experiments.

Ultrasonic vocalization recording and analyses

USV emission was recorded after each injection for all subjects. The recording chamber measured 25 cm wide x 18 cm deep x 18 cm height and was filled with dust-free beta-chip® bedding. No recording chamber was re-used between rats and every rat received a new recording chamber with fresh bedding. An UltraSoundGate CM16/CMPA (Avisoft Bioacoustics, Glienicke, Germany) condenser microphone (working frequency range 2-250 kHz) was used in the recording of emitted USVs. During a recording session the microphone was placed on top of the recording polycarbonate chamber (approximately 25 cm from the animal). The microphone was connected via an UltraSoundGate 416 USB audio device (Avisoft Bioacoustics) to a computer (Dell PC) and recordings made using multi-channel triggering hard-disk software (Avisoft RECORDER version 4.40). Acoustic data were recorded at a sampling rate of 250 kHz in 16-bit format. Analysis of USVs was done off-line using Avisoft SASLab Pro (version 4.40) and Sonotrack™ (Metris BV, The Netherlands) software (version 4.40).

The identification and characterization of 50 kHz USVs was accomplished in a manner described previously in several papers (Brudzynski et al., 1991; Brudzynski 2007, 2015; Thompson et al., 2006). Briefly, 50 kHz USVs had peak frequencies between 35 and 90 kHz and were typically less than 100 ms in duration. All spectrograms were generated using a fast Fourier transform (512 FFT-length, 100% Frame, Hamming window, and 75% time window overlap), at 488 Hz of frequency resolution. A bandpass filter was employed to reduce background noise (low and high cut-off frequencies of 25 and 90 kHz respectively). The two analysis programs were used for distinct and non-overlapping analyses. Avisoft SASLab generated spectrograms were manually screened for 50 kHz USVs and were used to calculate numbers of 50 kHz USV subtypes. Sonotrack generated spectrograms were utilized for preprogrammed automatic screening which determined number of USVs, mean call duration, and mean sound frequency of USVs. Cronbach's alpha for call detection between programs for saline (alpha = .702) and AMPH (alpha = .825) recordings indicates performance of the Sonotrack progam is comparable to a competent experimenter performing manual detection using Avisoft program.

Statistics

All statistical analyses were performed using SPSS Statistics (version 20, IBM Corporation). Paired t-tests were conducted on the sonographic characteristics of USVs emitted following saline or AMPH to investigate possible differences in average call duration and frequency. Direct mean comparisons for number of USVs counted from saline and AMPH recordings utilized the first 5 minutes of recording. To ensure optimal homogeneity of variances and to reduce positive skewness in the distributions L-maze

Latency (the sum of time across all testing sessions), Saline USVs (the sum of calls recorded after saline), and AMPH USVs (the average call rate per min recorded after AMPH) were all logarithmically transformed using the natural base of *e.* This was accomplished by taking the natural log of each individual score plus 1 (to correct for any zeroes in the data). Following transformation both the Kolmogorov-Smirnov and Shapiro-Wilk tests of normality were non-significant for all transformed variables and all relevant variables met the necessary assumptions associated with linear regression.

A MANOVA was conducted for food reward type (chocolate chip or fruit loop) as a between-subjects variable to assess if there were any differences across all the dependent measures of interest that could confound the main analyses. Roy's largest root was used as the multivariate statistic as it represents the maximal possible difference.

Where suitable, bias-corrected and accelerated bootstrap (BCa) confidence intervals are reported utilizing 1000 bootstrap samples. Effect sizes reported using Cohen's *d* for mean comparisons, with the general standard used for interpretation (small effect = 0.2, medium effect = 0.5, and large effect = 0.8).

Results

Descriptive statistics

Means and standard deviations of all continuous variables (with pre-transformation data) can be found in Table 2-1. Subject weight (measured in grams) refers to the average weight of the rat across the duration of all behavioural procedures. Sucrose Preference refers to the individual rat average across both testing days. L-maze Latency refers to the total number of seconds across all testing sessions for a rat to approach and consume an appetitive food stimulus. Average number of calls per minute

for both injection conditions is provided for 1st 5 minutes of recording for direct comparison.

Table 2-1. Descriptive statistics.

				Call rate (average number of calls/min for 5 min)	
	Subject Weight (g)	**Sucrose Preference (%)**	**L-maze Latency (s)**	**Saline**	**AMPH**
n	46	46	46	45	46
Mean	322.5	89.5	302.5	7.1	21.0
Std. Deviation	30.43	6.5	239.4	7.4	23.3
Minimum	258.7	65.9	35.0	0.0	0.0
Maximum	387.3	100.0	1193	31.2	82.0

USV characteristics between saline and AMPH

As expected, the AMPH injection induced a significantly greater number of USVs observed in the first 5 minutes of recording compared to that after saline ($t_{44} = 2.924$, $p = .005$, $d = 0.52$). To assess differences in USV emission characteristics between saline and AMPH recordings paired t-tests were conducted on the sonographic parameters of average call duration and sound frequency.

In 5 minutes of recording, AMPH-induced calls had a higher average sound frequency ($M = 59.51$ kHz, $SD = 8.7$ kHz) compared to the average sound frequency of saline-induced calls (M = 51.7 kHz, SD = 7.7 kHz). This difference was statistically

significant (t_{41} = -6.467, p < .001, d = 0.95). Additionally, a strong positive relationship was found between average sound frequency of AMPH and saline calls (N_{valid} = 42, r = .552, BCa 95% CI [0.188, 0.814], p < .001). Thus, individual rats with higher average sound frequency of calls in response to saline were more likely to show a higher average sound frequency of calls after AMPH.

In contrast to average sound frequency, there was no significant difference in average call duration between AMPH calls (M = 20.67 ms, SD = 6.22 ms) and saline calls (M = 19.14 ms, SD = 6.68 ms) found within the same 5 minutes of recording (t_{41} = -1.258, p = .216). There was also no significant relationship found for average duration between saline and AMPH calls (N_{valid} = 42, r = .256, BCa 95% CI [-0.66, 0.537], p = .102).

To investigate if the injection conditions differed in the proportion of subtypes among emitted 50 kHz USVs a random subset of the total sample was used for subtype analysis with non-callers excluded (n = 11). Individual differences in the proportion of specific subtypes were investigated by looking at the percent of total calls made up by flat, trill, and non-trill FM subtypes for each individual rat. A significant Injection x Subtype interaction was found (V = .554, $F_{2, 9}$ = 5.60, p = .026), indicating that there was a difference in percent of subtypes across injections. Post-hoc analysis found that on average AMPH increased the percent of the trill subtype (t_{10} = 3.492, BCa 95% CI [8.40, 25.66], p = .005, d = 1.01). There was no such difference between saline and AMPH recordings found for either flat or non-trill FM subtypes (see Figure 2-2).

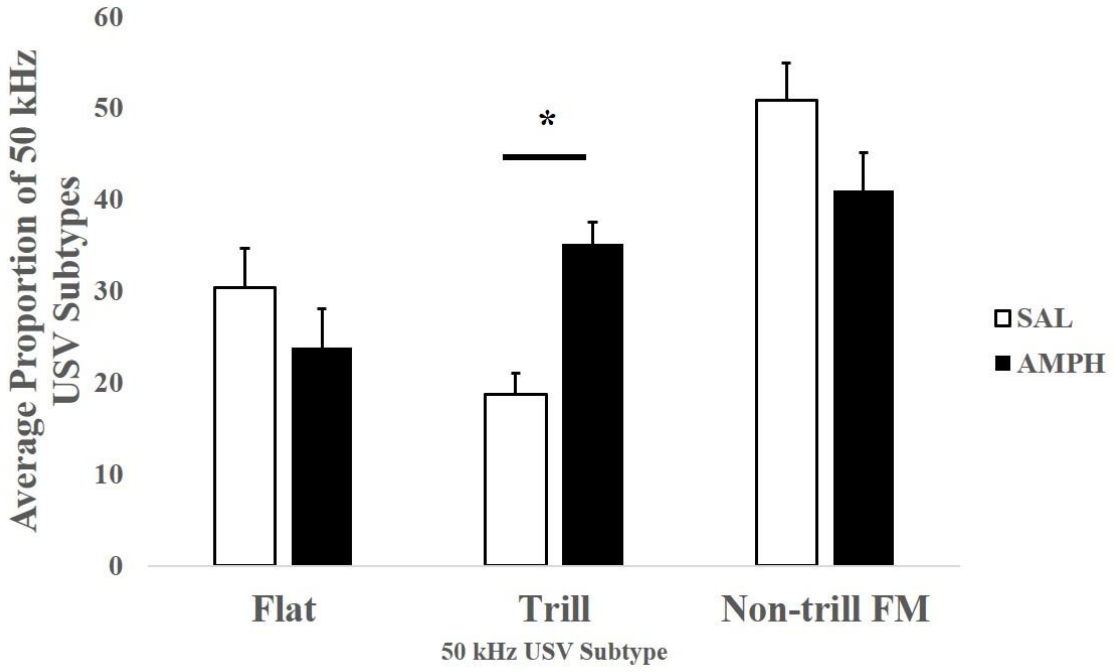

*Figure 2-2. Histogram illustrating the percent of total calls for each indicated 50 kHz USV subtype after saline (SAL, blank bars) and after amphetamine (AMPH, black bars). All calls for an individual rat = 100%. Results are represented as means ± SEM. * p < .05.*

Correlations

All correlation coefficients for relevant variables are included in Table 2-2. All variables except for sucrose preference have been logarithmically transformed. A significant positive relationship was found between number of USVs following saline injection (Saline USVs) and the rate of USV production after AMPH (AMPH USVs), such that an individual rat that emitted a greater number of calls after saline had a greater average rate of call emission following AMPH (Table 2-2). Also, latency to approach and consume palatable food in the L-shaped apparatus (L-maze Latency) was significantly negatively correlated to USV production after AMPH, such that an individual rat that was faster on average to approach and consume the food had significantly greater average rate of call emission following AMPH injection (Table 2-2). There was no significant relationship found between L-maze Latency and Saline USVs, indicating that the two variables relate to USV production following AMPH but not to each other. Sucrose preference was found to have no significant relationship with any of the relevant variables (see Table 2-2), and was thus not included as a predictor in the main analyses.

Table 2-2. Correlation matrix.

		1	2	3	4
1. Sucrose Preference	Pearson's r	—	-0.023	0.157	0.074
2. L-maze Latency	Pearson's r		—	-0.093	-0.388**
3. Saline USVs	Pearson's r			—	0.362*
4. AMPH USVs	Pearson's r				—

* p < .05, ** p < .01.

Main analyses

A MANOVA was conducted on all relevant dependent variables and L-maze food reward (chocolate chip or fruit loop) to ensure that the results on any given measure did not differ systematically by food reward. The MANOVA conducted used L-maze food reward as the grouping variable and latency to approach and consume in the L-maze, number of calls after saline, and call rate after AMPH as dependent measures. The results revealed no significant differences across all dependent measures between chocolate chip rats and fruit loop rats (θ = 0.054, $F_{3, 41}$= .732, p = .539). Thus, the two groups were collapsed and treated as one for all subsequent analyses.

A linear regression was conducted on call rate after AMPH using L-maze Latency and Saline USVs as predictors entered on a single step. The model significantly predicted the USV response to AMPH (R = .507, $F_{2, 43}$ = 7.42, p = .002). Both L-maze Latency and Saline USVs were significant predictors and together account for 25.7% of the variance in the response of rats to AMPH as measured via USV production (R^2 = .257). All B estimates of the model were significant (Table 2-3). Most importantly, the first-order partial correlations indicate that both predictors account for variance in AMPH USVs independently of each other (see Table 2-4, Figure 2-3, and Figure 2-4). L-maze Latency accounts for 14.6% of the variance in AMPH USVs when controlling for Saline USVs (see Figure 2-3); while Saline USVs accounts for 12.5% of the variance in AMPH USVs when L-maze Latency is controlled for (see Figure 2-4). Additionally, only 2.4% of the explained variance in AMPH USVs is shared between the predictors leaving 23.3% of AMPH USV variance accounted for by unique contributions of both predictors.

Table 2-3. Model parameters for main analysis regression.

	B (SE)	***95% CI for B***	***β***	***p (for Bs)***
Intercept	**4.509 (1.290)**	**1.909, 7.110**		**.001**
L-maze Latency	**-0.578 (.215)**	**0.067, 0.648**	**-.358**	**.017**
Saline USVs	**0.357 (0.145)**	**-1.009, -0.148**	**.327**	**.010**

Note. R^2 = .257

Table 2-4. Predictor coefficients for criterion of AMPH USVs.

	Partial	**Semipartial**
L-maze Latency	**-.382**	**-.356**
Saline USVs	**.353**	**.326**

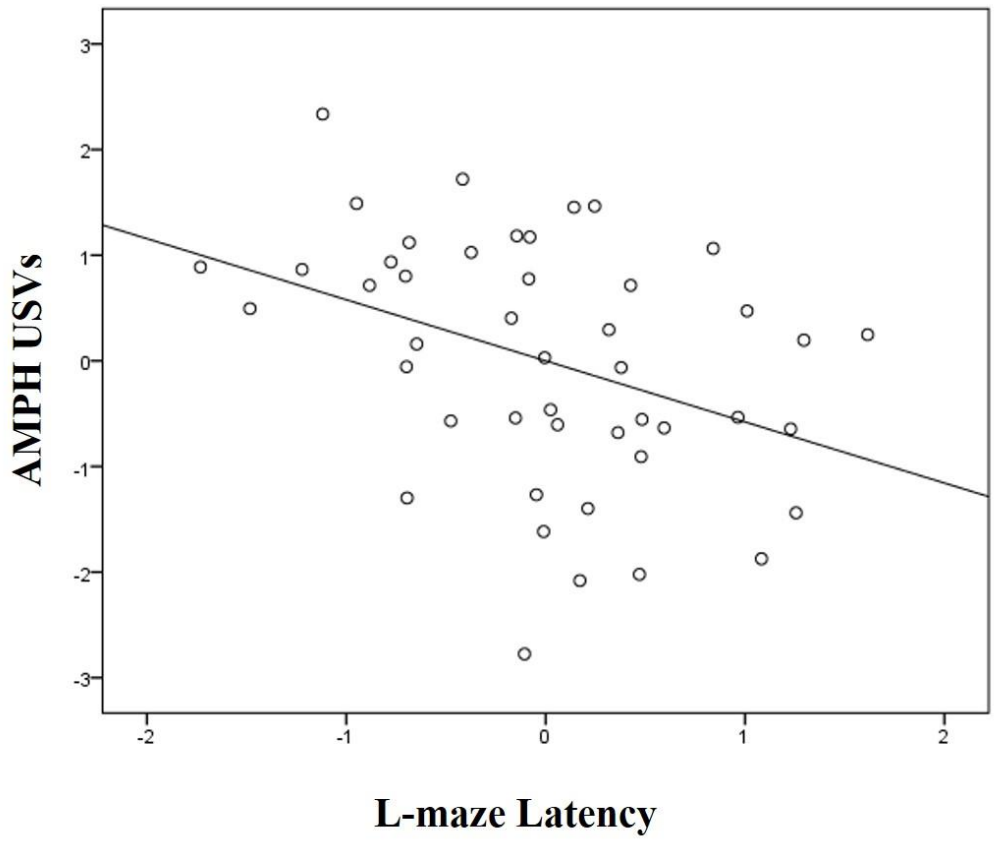

Figure 2-3. Scatterplot demonstrating partial relationship between log-transformed latency to approach and consume food reward in an L-maze and log-transformed rate of 50 kHz USV emission recorded after systemic amphetamine (AMPH USVs). The line represents the correlation between L-maze latency and AMPH USVs with saline USVs controlled for on both variables.

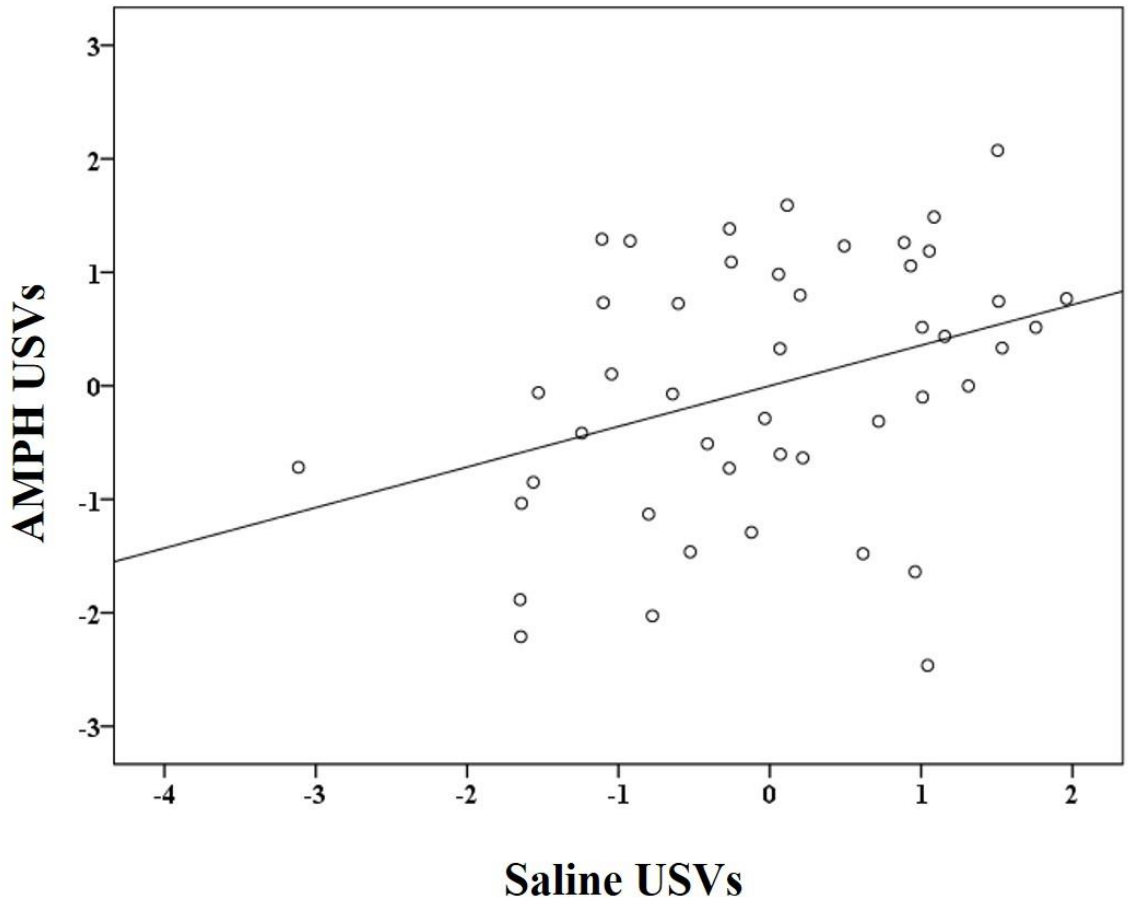

Figure 2-4. Scatterplot demonstrating partial relationship between log-transformed number of 50 kHz USVs recorded after saline and log-transformed rate of 50 kHz USV emission recorded after systemic amphetamine (AMPH USVs). The line represents the correlation between Saline USVs and AMPH USVs with L-maze latency controlled for on both variables.

Discussion

In this study, we employed a model of acute AMPH administration to induce 50 kHz calling in Long Evans rats. It was found that individual motivation to consume food reward and baseline predisposition to emit calls represent distinct predictors of the AMPH-induced call response. This finding supports the hypothesis that emission of 50 kHz USVs in response to acute administration of AMPH in the adult Long Evans rat indexes more than their individual characteristics associated with motivation for reward. 50 kHz calling in response to AMPH has been previously related to novelty and sensation seeking (NSS), conditioned place preference, and locomotor activity though with differentiable relations (Taracha et al., 2014; Garcia & Cain, 2016). The present experiment appears to be the first to relate an operational measure of food-reward motivation with acute AMPH administration using a within-subjects design. Our data support the idea that 50 kHz USVs induced by AMPH (without any sensitization) represent more than the reward-motivation phenotype of an individual rat. A rat's individual predisposition to emit 50 kHz calls (measured via the number of 50 kHz USVs emitted after saline) was not found to correlate with their motivation phenotype (measured via latency of the response in the L-maze). Moreover, given that the rats had no prior exposure to AMPH, the AMPH-induced USVs presumably represent a direct activation of the individual's underlying mesolimbic dopamine system and are not a product of anticipation. Thus the dissociable relation of the two predictors with AMPH-induced USVs may suggest the function of distinct subsystems within an individual's mesolimbic dopamine pathways.

Our results are consistent with the findings of Engelhardt et al. (2018) in discovering a positive relationship between baseline and AMPH-induced 50 kHz calling. In that study the authors employed juvenile rats while in the present paper this relationship is extended to adult rats. In contrast to these findings, Ahrens and colleagues (2013) failed to find any relationship between baseline predisposition to call and 50 kHz USV emission after acute application of AMPH. It is possible the results in our study are due in part to using a within-subjects design that allowed for greater statistical power to parse more subtle effects, similar to recent investigations of NSS and USV production (Garcia & Cain, 2016). However, Engelhardt and colleagues (2018) employed a high and low caller classification and still found a positive relationship between baseline and AMPH-induced 50 kHz USV emission. These different results may relate to dissimilarity in housing conditions and testing environment, such as the use of bedding, which has been found to be critical in promotion of 50 kHz USVs (Natusch & Schwarting, 2010).

As expected and consistent with relevant literature, AMPH induced a greater number of USVs compared to the number observed after saline. However, the literature regarding AMPH-induced 50 kHz USVs often fails to find any modulation of acoustic parameters (Wright et al., 2010; Simola et al., 2012; Taracha et al., 2012; Pereira, Andreatini, Schwarting, & Brenes, 2014). In contrast to this, we found evidence of difference in the average sound frequency between saline- and AMPH-induced calls, although no such effect on average call duration. This effect of AMPH-induced elevation of average sound frequency may reflect an increase in the proportion of FM 50 kHz USVs relative to flat calls between recordings, which is often found after AMPH (Ahrens et al., 2013). Consistent with the literature, we found an AMPH-induced increase in the

percent of total calls for the trill subtype of 50 kHz USVs. This finding supports the notion that the trill subtype is reflective of the magnitude of a given rat's response to systemic AMPH administration even when given acutely. The parameter of average sound frequency itself has been found to not undergo sensitization (Taracha et al., 2012), and thus may also be reflective of the acute action of systemic AMPH. It should be noted that there are a wide variety of 50 kHz USV classification systems (Takahashi et al., 2010; Wright et al., 2010). In this study we employed a relatively simple classification scheme focused on investigating the trill subtype relative to flat and other FM 50 kHz USV subtypes.

There was no significant relationship found between an individual rat's sucrose preference and any other experimental measure. Though it is tempting to derive a conclusion about the relative lack of relationship found between individual hedonic drive and AMPH-induced USVs, such a conclusion may not be warranted by the data. The concentration of sucrose in the current study (10 % w/v) was much higher than those previously associated with 50 kHz calling (1% solution; Mällo et al., 2007; 0.8%; Burgdorf et al., 2009). It is possible that the high sucrose concentration masked the more subtle behavioural relationship between sucrose preference and emission of 50 kHz USVs.

There were several limitations of the current study. Due to the within-subjects design and the acquisition of tissue following the AMPH recording there was no counter-balancing of the testing procedures across rats. Although this was advantageous for interpreting the USV results as their USV response to saline was not affected by prior exposure to AMPH it is impossible to ignore the possibility of carryover effects across

the behavioural procedures. Moreover, there are no data on what the relation of our current results are with the individual characteristics of sensitization to AMPH, nor do the results provide any information about a dose-dependent effect. In the current study, though standardized across all subjects, the USV recordings were limited in time (5 minutes for saline and 10 minutes for AMPH). There is empirical evidence on how critical the time-point of USV recording following systemic AMPH injections may be (Natusch & Schwarting, 2010). The start of recording in the current work was 10 minutes following injection (which should capture the peak of AMPH-induced calling according to the timeline found in Natusch & Schwarting, 2010); however, a longer recording time may have altered the outcome.

The dissociation of our predictors of AMPH-induced emission of 50 kHz USVs do indicate that an individual rat's predisposition to emit USVs ("cheerfulness"), both in relation to saline or a dopaminergic agonist such as AMPH, may be separate from its reward-related behavioural phenotype. AMPH-induced 50 kHz USVs appear in part to index the underlying operation of brain systems associated with the 'wanting' component of reward motivation. Individual behavioural differences among rats may depend, in part, on the different expression of these phenotypes and lead to dissociable patterns of emotionality. This was partially demonstrated by performing selective breeding of rats based on the magnitude and type of their vocalizations (Burgdorf et al, 2009; 2013). As a consequence of this selective breeding, juvenile rats with high or low levels of baseline emission of 50 kHz USVs had dramatically different responses to AMPH in adulthood (Brudzynski et al., 2011). However, our empirical evidence also indicates there is still

more left to elucidate and that the use of chronic exposure paradigms may mask certain characteristics associated with 50 kHz USV emission in rats.

Conclusion

The current study found evidence that approach latency to reward and emission of 50 kHz USVs after saline are unique behavioural predictors of 23.3% of the variance in 50 kHz USV emission observed after acute non-anticipated systemic AMPH. This acute AMPH was found to selectively alter acoustic parameters of 50 kHz USVs by elevating average sound frequency without affecting the duration of calls. AMPH also selectively increased the proportion of trill FM USVs, while not significantly affecting the proportion of flat or non-trill FM USVs. Collectively, these findings argue that 50 kHz USVs after acute systemic AMPH likely represent dissociable systems of emotionality operating within an individual rat. Ultimately, however, there is still much to uncover about the endogenous brain systems responsible for the emission of 50 kHz USVs induced by AMPH and their associated behavioural phenotypes.

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Rationale for Chapter 3

While often induced pharmacologically, 50 kHz USVs are commonly observed in a wide variety of non-pharmacological reward-related contexts. These contexts include sugary foods and drink, consumable ethanol, and social situations with conspecifics. As an overt expression of emotion, the emission of 50 kHz USVs is likely subject to context-specific modulation in behaviourally relevant ways. Aspects of this modulation may be masked by the use of powerful pharmacological inducers such as AMPH that activate a variety of neurochemical systems in the brain (Sulzer, Sonders, Poulsen, & Galli, 2005). As observed in chapter 2 and consistent with previous literature, AMPH produces an increase in the degree of frequency modulation and specifically the proportion of trill subtypes. A central aim of chapter 3 was to investigate and characterize 50 kHz USVs induced by different behavioural contexts and determine any context-specific associations with 50 kHz USV subtypes. This was done by directly comparing non-social and social contexts in their capacity to induce and modulate 50 kHz calling. In examining the proportion of 50 kHz USV subtypes across the varied behavioural contexts, the relation of frequency modulation as a signal of the magnitude of emotional arousal would be broadly investigated.

An additional primary aim of chapter 3 was to determine if the 50 kHz calling in these contexts was supported by a common underlying neurochemical system. Antagonism of dopamine signaling was utilized to establish whether regardless of context-specific modulation all 50 kHz USV emission was dependent on dopamine function.

Chapter 3: Non-pharmacological induction of rat 50 kHz ultrasonic vocalization: social and non-social contexts differentially induce 50 kHz call subtypes

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Author contribution: For this manuscript I conducted all aspects of the ethics approval process. For the experimental work the acquisition of data was assisted by two undergraduate students, all statistical analyses, however, were carried out by myself alone. I also carried out all writing and figure construction by myself with feedback and edits provided by Dr. Stefan Brudzynski.

Introduction

The emission of USVs in adult rats (*Rattus norvegicus*) has been conceptualized as a form of emotional expression and thus measurement of USVs has been used in a wide variety of experimental contexts (Burgdorf et al., 2008; Brudzynski, 2013, 2015; Wöhr & Schwarting, 2013; Rippberger, van Gaalen, Schwarting, & Wöhr, 2015). The two primary USV categories that have been differentiated and defined are the 22 kHz and the 50 kHz USV types. These categories are defined by both their sonographic appearance (acoustic features such as duration and sound frequency) and the context their emission is associated with (Brudzynski, 2007, 2009, 2013, 2015). The relatively low-frequency 22 kHz USVs typically occupy a narrow bandwidth (~3 kHz) and reflect a negative emotional state (Schwarting & Wöhr, 2012; Brudzynski, 2013). In contrast, the relatively high-frequency 50 kHz USVs have much greater variability in bandwidth (occurring as either non-modulated or frequency-modulated USVs) and reflect positive joy-like emotional states (Knutson, Burgdorf, & Panksepp, 1998, 1999; Brudzynski, 2007; Burgdorf & Moskal, 2010; Wöhr, 2018).

The 50 kHz USVs emitted by rats can be further subcategorized based on sonographic shape and acoustic characteristics (Brudzynski, 2015; Wöhr, 2018). There is no consensus on classification schemes with some researchers subdividing FM 50 kHz USVs into as many as 14 different subtypes and some as few as two (Wright, Gourdon, & Clarke, 2010; Burgdorf, Panksepp, & Moskal, 2011). The broad distinction between FM and flat 50 kHz USVs has, however, been used effectively across a range of experimental work (Burgdorf et al., 2008, 2009; Taracha et al., 2012; Wright, Dobosiewicz, & Clarke, 2013; Taylor, Urbano, & Cooper, 2017). It has been suggested that FM calls represent a

general positive emotional state while flat calls represent a social contact call (Wöhr, Houx, Schwarting, & Spruijt, 2008; Burgdorf & Moskal, 2010; Burgdorf et al., 2011). There is evidence, however, that FM calls may serve a communicative function within the social play of juvenile rats (Burke, Kisko, Euston, & Pellis, 2018). One of the most well supported 50 kHz USV subtypes is the trill call which is characterized by a fast sine wave-like oscillation of call frequency (Brudzynski, 2015). The proportion of trill calls is selectively increased in response to dopaminergic agonists (e.g., AMPH; Wright et al., 2010; Mulvihill & Brudzynski, 2018), and shows sensitization patterns whereas flat and other FM subtypes do not (Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009); however the behavioural relevance of this call type remains unclear. Burke et al. (2017) investigated behavioural associations of specific 50 kHz call types in juvenile rats and found evidence that suggests trill calls may be reflective of a high magnitude of general arousal. The recording of this call type may thus may be expected in a variety of behaviourally activating situations.

Among non-pharmacological situations, 50 kHz USVs have been extensively mapped onto contexts associated with mating and sexual behaviour (Barfield, Auerbach, Geyer, & McIntosh, 1979; McIntosh & Barfield, 1980). Adult males will emit 50 kHz USVs without any prior successful sexual contact (Bialy, Rydz, & Kaczmarek, 2000), and 50 kHz USVs have been found to elicit social approach (Willadsen, Seffer, Schwarting, & Wöhr, 2014) and sexual behaviour in females (McIntosh, Barfield, & Geyer, 1978). Remarkably, there is little evidence to date on the specific impact of exposure to a female on emission of 50 kHz USV subtypes in male rats. Early descriptions showed that 50 kHz USVs emitted during copulatory behavioural sequences

were of complex and frequency-modulated calls (Sales, 1972; Barfield et al., 1979). More recently, Burgdorf et al. (2008) found positive correlations between FM calls (including trills) and appetitive behaviours during mating in isolate housed 3-month old males. However, these results reflect calls emitted with the behavioural activation concomitant with the active engagement in the mating context. Such recorded calling may be qualitatively distinct from male rat USVs emitted in response to a female without the act of engagement. In response to an anesthetized female (either ovariectomized or proestrous) male rats did produce a greater number of FM and flat calls to the proestrous female in socially housed animals (Inagaki, Kuwahara, Tsubone, & Mori, 2013). The relative proportions of FM and flat calls was not reported however.

In the broader non-sexual social context, extensive research has investigated the communicatory role of 50 kHz USVs among male conspecifics (for reviews see: Seffer, Schwarting, & Wöhr, 2014; Wöhr, 2018). These calls appear to promote playful contact in juveniles and facilitate non-aggressive encounters in adults (Kisko, Himmler, Himmler, Euston, & Pellis, 2015; Kisko, Wöhr, Pellis, & Pellis, 2017). It has been suggested that these 50 kHz calls may index social motivation. Juvenile rats emit the greatest number of 50 kHz USVs in anticipation of playful contact and this calling may be enhanced by social deprivation (Knutson et al., 1998; Willey & Spear, 2013; Himmler, Kisko, Euston, Kolb, & Pellis, 2014). Adult rats develop anticipatory vocalizations to re-union with their cage-mate (Willey & Spear, 2012), and Wöhr et al. (2008) found that separation from cage-mate appeared to elicit a specific ratio of flat relative to FM calls, with a high rate of flat 50 kHz USV emission in naïve rats observed after isolation.

Consumable food and ethanol (EtOH) rewards have been used in a variety of studies investigating 50 kHz USV emission (Willey & Spear, 2013; Brenes & Schwarting, 2014, 2015; Buck, Malavar, George, Koob, & Vendruscolo, 2014a; Garcia, Jorgensen, Sprick, & Cain, 2017). The motivation for these consumable rewards appears to share a common mechanism with that for emission of 50 kHz USVs, namely mesolimbic dopamine (Burgdorf Knutson, & Panksepp, 2000; Buck, Vendruscolo, Koob, & George, 2014b; Brenes & Schwarting, 2015). Indeed, there is even evidence of behavioural cross-tolerance between food reward and AMPH-induced 50 kHz USV emission (Brenes & Schwarting, 2015). In 24 h recordings, Opiol et al. (2015) found that rats appear to emit high rates of 50 kHz USVs in anticipation of a meal with different temporal profiles for FM and flat calls. These authors also found evidence of flat calls occurring in socially isolated rats and rates of flat call emission specifically increasing in association with mealtime occurrence. These results appear consistent with earlier reports of an association of flat 50 kHz USVs and feeding behaviour (Takahashi, Kashino, & Hironaka, 2010).

In the present study we sought to make a direct experimental comparison of the emission of 50 kHz USVs induced by several types of commonly used consumable and social reward contexts. In the aim of providing behavioural relevance to the proportion of a given 50 kHz USV emitted by adult rats in non-pharmacological contexts, we measured flat, trill, and non-trill FM 50 kHz USVs across multiple sessions in response to one of four stimulus conditions: Fruit Loops, EtOH, exposure to a female, or a reunion with a cage-mate. We hypothesized that given the suggested communicatory role of 50 kHz USVs the social conditions would more robustly induce call emission than the non-social

conditions. Additionally, it was hypothesized that the four conditions would differ in their proportions of USV subtype, with the consumable reward conditions having a greater proportion of flat calls than the social. The cage-mate and female conditions were expected to differentially induce 50 kHz USV subtypes with the cage-mate condition potentially inducing greater contact calls (flats) than FM calls while the female condition would induce a greater amount of FM. We also used injections of the dopamine receptor antagonist haloperidol (HAL) to challenge the stimulus-induced 50 kHz USV production. It was hypothesized that all stimulus-contexts would share a common association with the activity of the mesolimbic dopamine system and thereby have a significant reduction in calling after antagonist administration.

Methods

Subjects

A total of 41 Long Evans rats (Charles River Laboratories, Saint-Constant, QC, Canada) were used in this study (32 male rats used as experimental subjects, 8 males and 1 naturally cycling female used as stimulus animals). At the start of the study, experimental subjects had an average body weight of 301 g (*SD* = 18.4 g, range = 80 g) and were approximately 60 days old. At the end of the experiment subjects had an average body weight of 444 g (*SD* = 34.5 g, range = 184 g) and were approximately 85 days old. In accordance with Brock University protocols for laboratory handling, all animals were housed in pairs in polycarbonate cages (48 x 27 x 20 cm) with a plastic tube inside for hiding. Where applicable, cage-mate pairs shared the same condition. The housing room had controlled room temperature (23° C ± 2° C) and humidity (40-60%). Subjects were housed with a maintained 12:12 h light/dark cycle and *ad libitum* access to

food pellets and water. Animals were gently handled once before the initial behavioural procedure. This preliminary handling consisted of transfer from home-cage to the testing apparatus and vice-versa once for all subjects (including experimental and stimulus animals). All research protocols were approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

Materials

A two-chamber apparatus made of solid-opaque washable plastic (Technical Services, Brock University) was used for all behavioural context exposures and USV recordings. Both chambers were of equal size (20 x 20 x 25 cm) and were capable of being separated by a removable partition. The partition was either solid-opaque plastic (used for alimentary conditions) or had eight small holes (0.5 cm in diameter) located in two rows 1.5 cm and 3 cm from the bottom (used for social conditions).

USVs were recorded using an UltraSoundGate CM16/CMPA (Avisoft Bioacoustics, Glienicke, Germany) condenser microphone (working frequency range 2-250 kHz) located on top of the recording apparatus on a metal grate (approximately 25 cm from the animal). The microphone was connected via an UltraSoundGate 416 USB audio device (Avisoft Bioacoustics) to a computer (Dell PC) and recordings were made using multi-channel triggering hard-disk software (Avisoft RECORDER version 4.40). Acoustic data were recorded at a sampling rate of 250 kHz in 16-bit format. Analysis of USVs was done off-line using Avisoft SASLab Pro (version 4.40).

Procedure

Experimental subjects were randomly split into 4 groups (8 rats in each experimental condition) to investigate the effect of four USV-inducing stimulus conditions (two social and two alimentary in nature). The social conditions were comprised of exposure to either a female conspecific or a male cage-mate conspecific. The alimentary or 'food-reward' conditions comprised presentation of either Fruit Loops® (sweetened, fruit-flavored cereal, Kellogg's Company, Battle Creek, MI, U.S.A.) or an ethanol solution (2% EtOH v/v).

Prior to any behavioural procedure all subjects were habituated to brief and gentle handling and to the apparatus to avoid novelty-induced USVs. The non-social condition animals were additionally habituated through overnight exposure to their respective food stimulus (Fruit Loops or EtOH). This overnight exposure consisted of placement of either 25 ml of EtOH solution or 8 Fruit Loops into the home-cage for the duration of the night phase and it was repeated for three consecutive nights. During this habituation the EtOH solution was sweetened with 15 mg of sucrose (15% solution w/v) to induce initial consumption, though during testing unsweetened-EtOH was used. The animals in the social condition received no habituation exposure to their respective experimental stimulus.

Following the necessary initial habituation, all 32 experimental animals underwent baseline recordings (5 min) in the apparatus where they were simply placed in one chamber of the apparatus and their USV production was recorded. Following this baseline recording the non-social condition animals underwent procedural training whereby they were repeatedly exposed to the apparatus and their respective food stimulus until they would rapidly (latency < 30 s) consume the stimulus upon access. For the

subjects in the Fruit Loop condition this took four training sessions while for subjects in the EtOH condition this took seven training sessions. The social condition animals received general habituation to the apparatus but no training to expect any rat stimulus. There were four standard recording sessions where each experimental rat was exposed to their respective stimulus in the apparatus and their individual USV production was recorded. The 5th and 6th recording sessions were paired with pre-injections of either HAL (0.5 mg/kg s.c. in 1% lactic acid) or vehicle (in counterbalanced order). Injectable solutions were prepared fresh the day of recording.

A 7th recording session was conducted using the cage-mate stimulus animals as stimulus controls. These rats were exposed to the apparatus in the same manner as the experimental groups for the same duration with no stimulus present and their USV emission was recorded. On every recording day the order of exposure to stimulus was counter-balanced across rats and the apparatus was extensively cleaned between each subject using a 70% EtOH solution.

Stimulus conditions

The female exposure condition consisted of a modified procedure derived from McGinnis and Vakulenko (2003). This procedure involved the experimental rat being placed into the apparatus with a female Long Evans rat present in the adjacent chamber separated by a perforated partition. The two rats were in the apparatus for no more than 5 min, at which point the female conspecific was removed from the apparatus and the partition was also removed to allow the experimental male to explore for 5 min. It was during this latter 5 min that USVs were recorded. Only one female rat was used as stimulus for all experimental rats in the female exposure condition.

The cage-mate exposure condition consisted of the experimental rat being placed into the apparatus while its corresponding male cage-mate was present in the adjacent chamber separated by a holed partition. The two rats were separated (placed into waiting cages with home-cage bedding split equally between them) for 1.5 h before being placed into their respective apparatus chambers. The cage-mate stimulus animals were only used as behavioural stimuli and were not subjected to any experimental manipulation concurrent with this procedure. The two rats were separated by the partition for 5 min, at which point both the cage-mate conspecific and partition were removed and the experimental male could explore the adjoining chamber for 5 min. It was during this latter 5 min that USVs were recorded.

The two food-reward conditions consisted of the experimental rat being placed into the apparatus for a 5-min anticipation period with the solid partition blocking access to their respective food-reward (i.e., either Fruit Loops or 2% EtOH). After 5 min had elapsed the partition was removed allowing the experimental rat to access and consume the food-reward for no longer than 5 min. USV production in both 5-min periods was recorded.

Data analysis

USV calls were analyzed and the identification and characterization of USVs was accomplished in a manner as described previously in several papers (Brudzynski, 2009, 2015; Thompson, Leonard, & Brudzynski, 2006). Briefly, 50 kHz USVs had peak frequencies between 35 and 90 kHz, were typically less than 100 ms in duration, and had varying degrees of frequency modulation. 22 kHz USVs were rare or absent but would be identified by having a low peak frequency (20 – 30 kHz), long duration, and with

constant frequency. Given the virtual absence of 22 kHz USVs this call type was omitted from the analysis. Avisoft SASlab-generated spectrograms were manually screened for 50 kHz USVs and were used to calculate sonographic parameters of peak frequency (in kHz), duration (in ms), and bandwidth (in Hz) of individual calls in addition to determining call subtypes. USV subtype determination was based on sonographic shape (for sample sonograms see Figure 3-1). 50 kHz calls were classified into the flat subtype if they appeared to have a relatively constant frequency. If the calls were frequency-modulated they were classified as either trill or non-trill subtypes. All spectrograms were generated using a fast Fourier transform (512 FFT-length, 100% frame, Flat Top window, and 75% time window overlap), at 488 Hz of frequency resolution. Manual screening of 50 kHz USVs was accomplished by three trained experimenters with an inter-rater reliability exceeding 99% (Intraclass correlation coefficient = .999, 95% CI: .993, 1.00).

Statistics

All statistical analyses were performed using SPSS Statistics (version 20, IBM Corporation). Where suitable, repeated-measures analyses of variance (ANOVAs) were used to assess the effect of the various behavioural stimuli on eliciting 50 kHz USV emission across multiple recording sessions. In every repeated-measure analysis the recording sessions served as within-subject factor and the stimulus groups served as between-subject factor. No subject exclusions took place for analyses involving call rate (valid n = 32). Prior to any analyses on acoustic parameters or call subtype proportions associated with 50 kHz USVs any subject with an absence of calls in either baseline or first session recordings was excluded from the respective analysis. This resulted in an

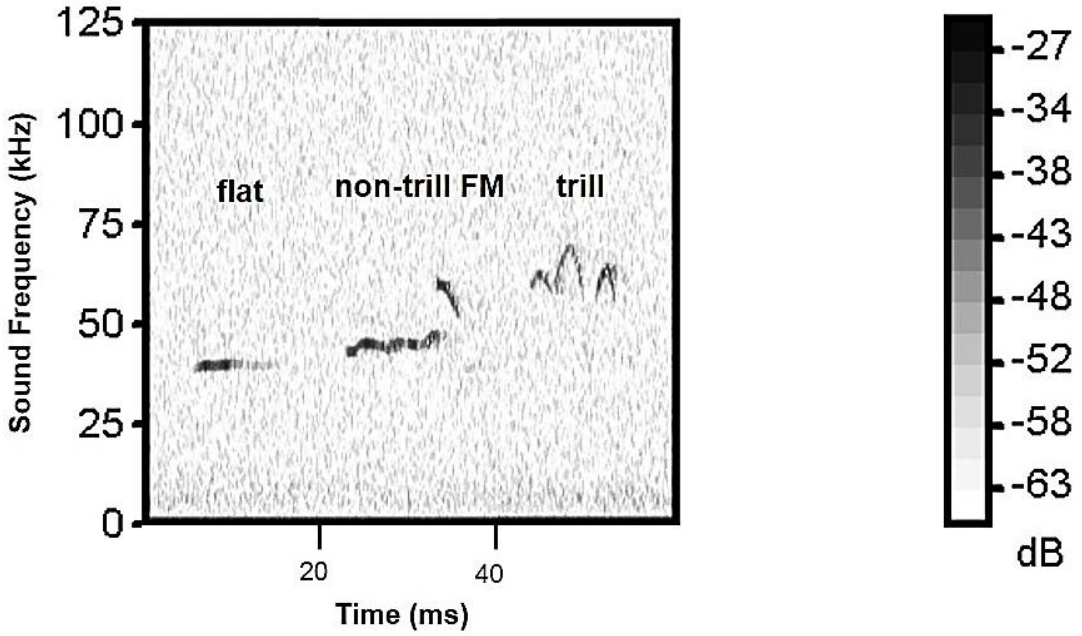

Figure 3-1. Representative spectrographic display of the 50 kHz USV subtypes used with examples of flat, non-trill FM and trill calls (represented left to right). Non-trill FM calls were classified as all FM calls not strictly fitting the trill sonographic shape. Intensity of calls is expressed in a gray scale labeled in dB.

exclusion of 12 subjects (remaining valid n = 20) for analyses involving baseline and an exclusion of 2 subjects (remaining valid n = 30) for analyses involving only session one. To assess the effect of behavioural stimuli across the multiple dependent measures of acoustic parameters multivariate ANOVAs were employed. Moreover, a multivariate analytic approach was taken for analyses of 50 kHz USV subtype proportions after stimulus exposure due to the high correlative nature of these variables. Pillai's trace (V) was reported as the multivariate statistic wherever appropriate. Significant main effects and interactions were investigated using either Bonferroni or Games-Howell corrections for post-hoc tests where suitable.

Results

Characteristics of 50 kHz USV emission at baseline

Since many of the research questions in the current study depend on a difference from baseline it was necessary to determine if the experimental groups differed in their calling at baseline. This calling was recorded prior to condition-dependent stimulus training and was analyzed as number of calls per minute. To address this, a one-way ANOVA was conducted on call rate during baseline recording across the four stimulus groups. No significant difference was found across the stimulus groups ($F_{3, 28} = 1.35$, $p = .279$).

To investigate possible differences in acoustic parameters or the proportion of 50 kHz USV subtypes across the experimental groups, any subjects with no calls emitted at baseline were excluded from analyses (12 subjects excluded, valid n = 20). A MANOVA conducted on 50 kHz USV acoustic parameters at baseline found no significant difference ($V = .207$, $F_{9, 48} = .395$, $p = .932$) across all four groups. Thus, there was no

significant difference across groups for average peak frequency ($F_{3, 16}$ = .404, p = .752), average call duration ($F_{3, 16}$ = .052, p = .984), and average bandwidth of calls ($F_{3, 16}$ = .412, p = .412).

A repeated measures ANOVA on the proportion of 50 kHz USV subtypes across the four groups during the baseline recording found no significant difference ($F_{6, 32}$ = .290, p = .937). Thus, at baseline, all stimulus-conditions had comparable subtype proportions for observed flat, trill, and non-trill FM 50 kHz USVs.

Effects of stimuli on call rate across experimental groups

A preliminary repeated-measures ANOVA on the number of 50 kHz USVs/min for all groups upon initial stimulus exposure (first session) compared with baseline indicated a significant main effect of stimulus exposure ($F_{1, 28}$ = 24.50, p < .001) and group ($F_{(3, 28)}$ = 4.23, p = .014), with a significant interaction of Session x Group ($F_{3, 28}$ = 5.84, p = .003). Thus, there was successful induction of 50 kHz USVs via the behavioural stimulus exposure though differentially across groups. Games-Howell post-hoc tests indicated that the social conditions of female exposure and cage-mate exposure were not significantly different from each other in 50 kHz call rate induced upon initial exposure to rat stimulus (p = .862). However, the initial exposure (first session) to a female induced a greater 50 kHz call rate than both non-social rewards (food: p = .009; EtOH: p = .025). Initial re-union exposure to cage-mate had no such difference from either of the non-social food rewards in observed 50 kHz call rate (see Figure 3-2).

To investigate the effect of the different behavioural stimuli on 50 kHz USV production across the four recording sessions a repeated measures ANOVA was run using the baseline plus four recorded sessions as within-subjects factor and the four

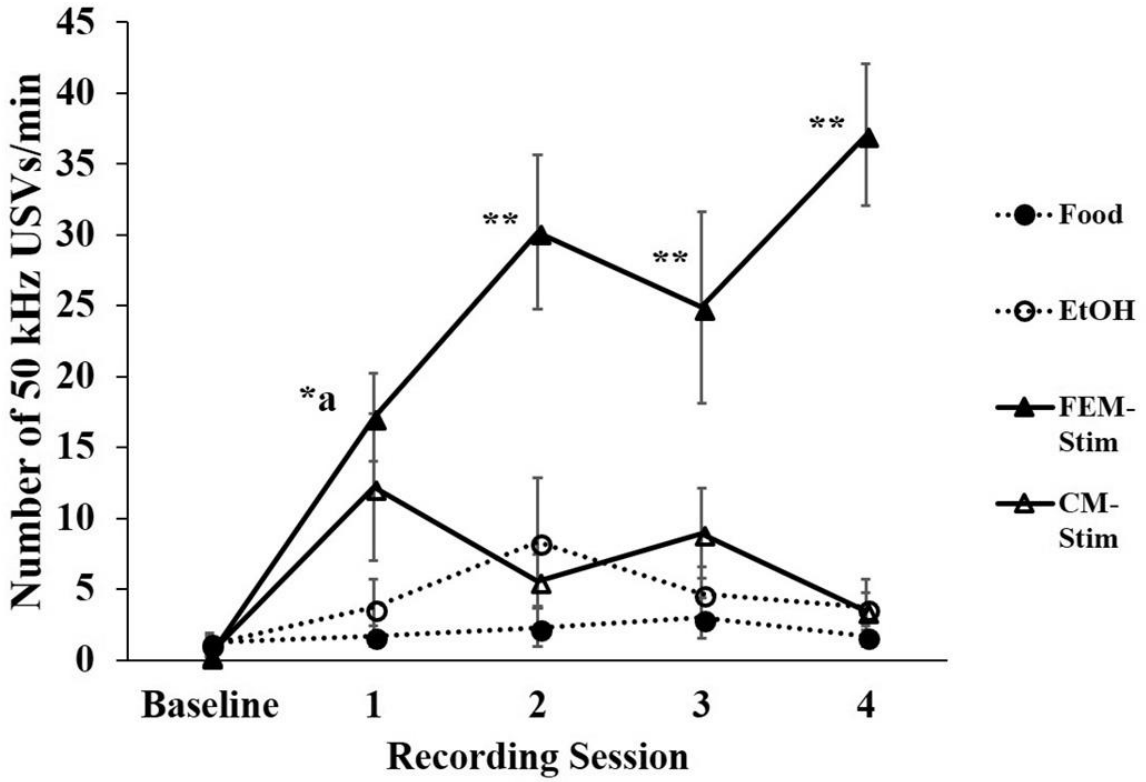

*Figure 3-2. Average number of 50 kHz USVs per min recorded from each experimental group (each group n = 8) across baseline and the four standard recording sessions. Fruit Loop stimulus (Food) and ethanol stimulus (EtOH) were the two consumable food reward contexts used and are plotted as dash lines. Female stimulus (FEM-Stim) and same-sex cage-mate stimulus (CM-Stim) were the two social contexts used and are plotted as solid lines. Results are represented as means ± SEM. No significant differences between groups found at baseline. The asterisk (*****) indicates female exposure significantly different from food and ethanol at p < .05 in first session. The (**a**) indicates female exposure in first session significantly different compared with baseline with p = .001. The double asterisk (**) indicates female exposure significantly different from all other groups at p < .01.*

experimental groups as the between-subjects factor (5 x 4 mixed design). Mauchly's test indicated that sphericity could be assumed ($X^2_9 = 13.79$, $p = .131$). There was a significant main effect found for both session ($F_{4, 112} = 11.32$, $p < .001$), and group factor ($F_{3, 28} = 22.06$, $p < .001$), indicating significant differences across both the five recording sessions and experimental groups in observed 50 kHz call rate. In addition, a significant overall Session x Group interaction was observed ($F_{12, 112} = 6.75$, $p < .001$). Simple *a priori* contrasts comparing all four recording sessions with baseline indicated that all four recording sessions had significantly greater call rate compared with baseline (first session: $F_{1, 28} = 24.50$, $p < .001$; second session: $F_{1, 28} = 35.01$, $p < .001$; third session: $F_{1, 28} = 23.95$, $p < .001$; fourth session: $F_{1, 28} = 57.16$, $p < .001$). Thus, for every recorded session with stimulus presentation a greater than baseline 50 kHz call rate was induced across the stimulus groups. Additionally, there was a significant Session x Group interaction observed for each recording session relative to baseline (first session: $F_{3, 28} = 5.84$, $p = .003$; second session: $F_{3, 28} = 12.58$, $p < .001$; third session: $F_{3, 28} = 7.02$, $p = .001$; fourth session: $F_{3, 28} = 37.85$, $p < .001$). Post-hoc Games-Howell pairwise comparisons indicated that the female exposure group significantly differed from all other experimental groups and drove these interactions (all $p < .01$; see Figure 3-2).

Bonferroni-corrected paired t-tests between initial session and baseline for each of the stimulus groups found only the female stimulus was effective in inducing greater call rate than baseline ($t_7 = 5.44$, $p = .001$). Exposure to cage-mate, Fruit Loops, and EtOH appeared to fail to significantly increase 50 kHz USV emission over baseline. Additionally, these non-female behavioural stimuli did not gain any significant effect across sessions, as the only experimental group to show an appreciable sensitization of

call rate was the female exposure group (which showed peak call rate in the fourth recording session). Therefore, it should be concluded that the female stimulus was the only effective stimulus in inducing greater 50 kHz USV call rate and was responsible for both main effects and the observed interaction (see Figure 3-2).

Effects of stimuli on 50 kHz USV acoustic parameters across experimental groups

A MANOVA conducted on 50 kHz USV acoustic parameters observed during the first exposure session (valid n = 30) found no significant difference (V = .207, $F_{9, 48}$ = .395, p = .932) across all four experimental groups. There was no significant difference across the groups for average peak frequency ($F_{3, 26}$ = .780, p = .516), average call duration ($F_{3, 26}$ = .416, p = .743), and average bandwidth of calls ($F_{3, 26}$ = 1.36, p = .277). Thus, observed 50 kHz USVs appeared to have comparable sonographic characteristics across all experimental groups upon exposure to their respective stimulus. There was also no significant difference found between the stimulus exposure session and baseline on any of the acoustic parameters (V = .098, $F_{1, 16}$ = 1.74, p = .205). The 50 kHz USVs elicited in the behavioural stimulus context thus do not appear to be sonographically different than those emitted under baseline conditions (see Figure 3-3).

Effect of haloperidol on stimulus-induced 50 kHz USV call rate

A repeated-measures (3 x 4 mixed design) ANOVA conducted on baseline, vehicle+stimulus, and haloperidol+stimulus recording sessions for all experimental groups was used to investigate the effect of dopaminergic antagonism on stimulus-induced 50 kHz USV emission. Mauchly's test indicated that sphericity could not be assumed for session factor (X^2_2 = 76.91, p < .001, therefore Greenhouse-Geisser corrected tests are reported (ε = .52).

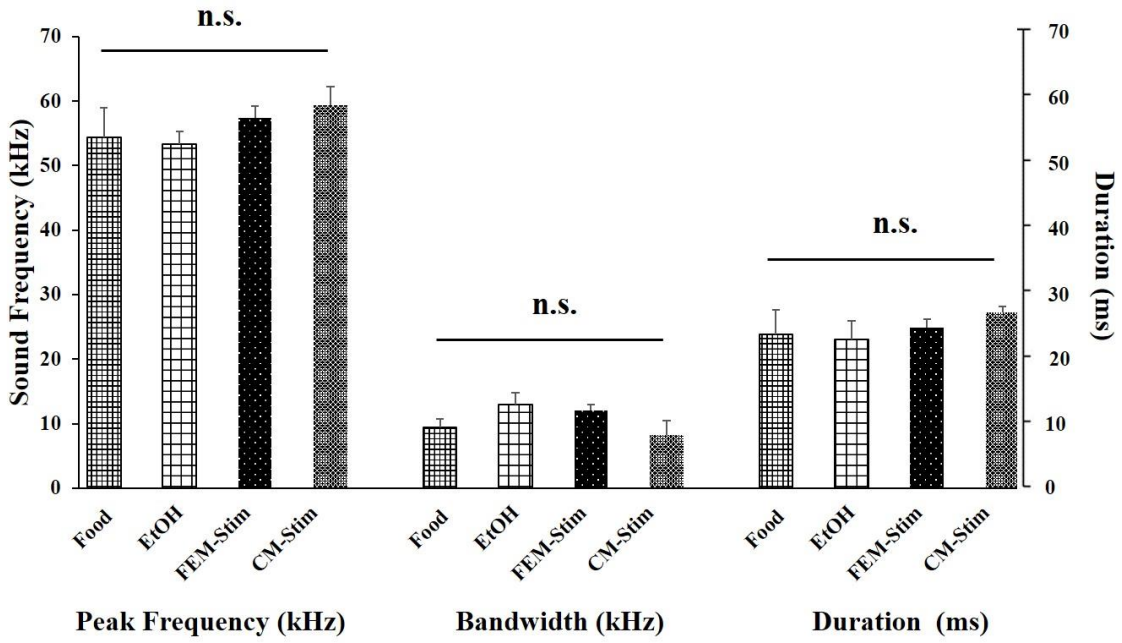

*Figure 3-3. Comparison of acoustic parameters across all four experimental groups for 50 kHz USVs recorded in first stimulus exposure session. A likewise comparison of acoustic parameters at baseline appears similar, with no significant differences across all four groups. Fruit Loop stimulus (Food) and ethanol stimulus (EtOH) were the two consumable food reward contexts used and female stimulus (FEM-Stim) and same-sex cage-mate stimulus (CM-Stim) were the two social contexts used. Peak frequency in kHz, bandwidth in kHz, and duration in milliseconds were measured for individual single calls. Bandwidth is expressed on the same axis as peak frequency in kHz. No significant differences were found between any of the groups for all acoustic parameters measured. Results are represented as means ± SEM. Use of (**n.s.**) denotes no significant differences across experimental groups within a given parameter (all $p > .05$).*

A significant effect of session was found ($F_{1.03, 28.84} = 29.62$, $p < .001$), with *a priori* contrasts indicating that with pre-treatment of vehicle the exposure to behavioural stimuli was able to significantly induce 50 kHz calling relative to baseline ($F_{1, 28} = 27.49$, $p < .001$). Moreover, pre-treatment with HAL prior to behavioural stimulus exposure significantly reduced 50 kHz USV production relative to vehicle (lactic acid) pre-treatment ($F_{1, 28} = 35.77$, $p < .001$). An overall Session x Condition interaction was found to be significant ($F_{3.09, 28.84} = 4.78$, $p = .008$). The experimental groups differed in their 50 kHz USV call rate in the vehicle condition relative to baseline ($F_{3, 28} = 5.01$, $p = .007$) as well as between HAL and vehicle sessions ($F_{3, 28} = 4.13$, $p = .015$). Games-Howell pairwise comparisons indicate that in vehicle condition the only two groups that were significantly different were the female and cage-mate stimulus groups ($p = .031$); however, in the HAL condition no group differences were observed (see Figure 3-4).

Effect of behavioural stimuli on 50 kHz USV subtype proportions across experimental groups

Due to the higher rate of non-callers observed at baseline recording this session did not serve as a viable control measure. Alternatively, a quasi-control was employed using the non-experimental cage-mate partners recorded in a 7th session as an additional between-subjects measure for analyses of subtype proportions. This group of animals received exposure to the apparatus with no stimulus present.

A MANOVA conducted on flat, trill, and non-trill FM coded 50 kHz USV proportions across the four experimental groups after initial exposure to stimulus (valid n = 30) found a significant overall effect of group ($V = .807$, $F_{9, 78} = 3.19$, $p = .002$; for visual examples of the call types see Figure 3-1). This indicates that the observed

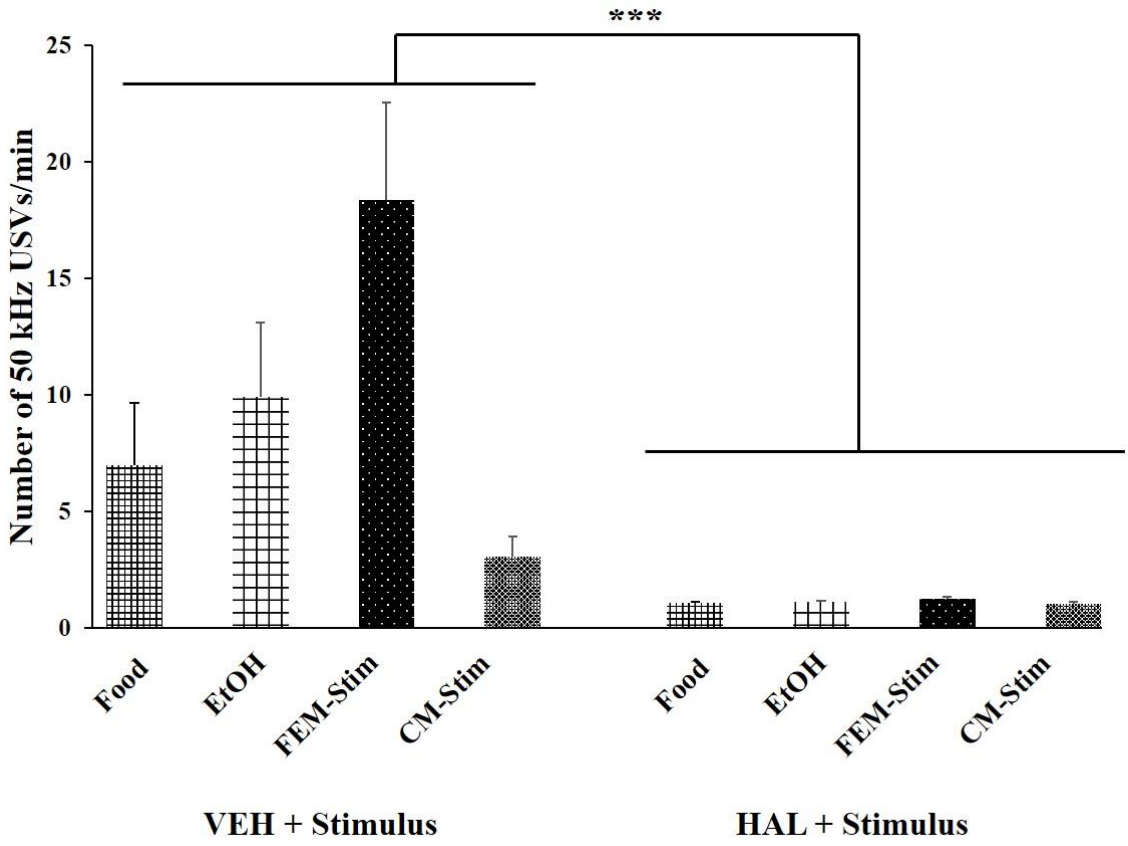

Figure 3-4. Average number of 50 kHz USVs per min across all four experimental groups recorded after vehicle pre-treatment (VEH + Stimulus) or dopamine antagonist haloperidol (HAL + Stimulus). Fruit Loop stimulus (Food) and ethanol stimulus (EtOH) were the two consumable food reward contexts used and female stimulus (FEM-Stim) and same-sex cage-mate stimulus (CM-Stim) were the two social contexts used. Pre-treatment condition represents counter-balanced within-subjects variable. For illustration purposes only, data was linearly transformed (all scores +1) to allow for the data close to zero to be visualized. Presence of (*******) indicates main effect of drug. All groups had call rate significantly reduced by haloperidol ($p < .001$). Results are represented as means ± SEM.

proportion of each subtype was not equal across the groups. There was a significant difference across groups for average proportion of flat calls ($F_{3, 26} = 17.17$, $p < .001$), trill calls ($F_{3, 26} = 3.64$, $p = .026$), and non-trill FM ($F_{3, 26} = 7.39$, $p = .001$). The inclusion of the control group did not change these omnibus results (see Figure 3-5).

Planned simple contrasts between each experimental group and the control on the average proportion of the flat call subtype indicated that the non-social conditions (food and EtOH) both had an increased proportion of flat 50 kHz USVs (both $p < .05$). Neither social experimental group had a significantly different proportion of flat calls from the control. Bonferroni-corrected post-hoc comparisons among the groups indicated that Food and EtOH conditions did not differ in their proportion of the flat subtype from each other ($p = .573$). However, both non-social conditions had a significantly greater proportion of flat calls than the female exposure group (both $p < .05$), though only the food condition differed from cage-mate ($p = .001$). Female and cage-mate conditions did not differ from each other in their proportion of flat calls ($p = 1.0$; see Figure 3-5).

Planned simple contrasts between each experimental group and the control on the average proportion of the trill call subtype indicated that only the female group significantly differed from the control ($p = .039$). The female stimulus significantly increased the proportion of trill calls recorded compared to the control. Bonferroni-corrected post-hoc comparisons across the experimental groups indicated that the only two groups that were significantly different from each other in the proportion of trill calls were food and female exposure groups ($p = .021$). The female stimulus induced a significantly greater proportion of trill calls compared with the food exposure condition (see Figure 3-5).

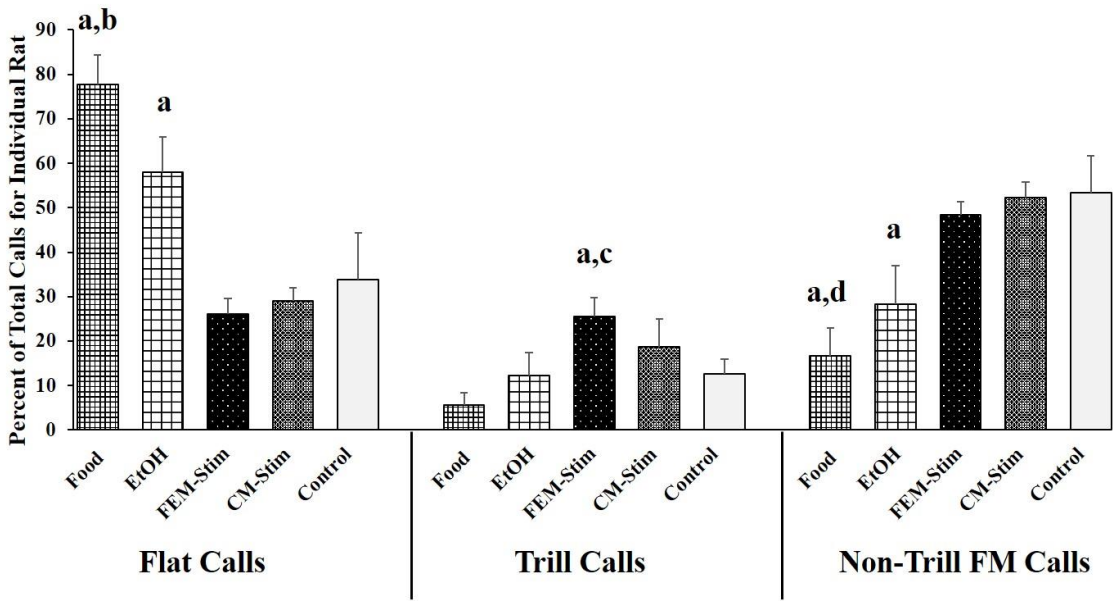

*Figure 3-5. Comparison of 50 kHz USV proportions across all four experimental groups recorded during first exposure session plus control for flat, trill and non-trill FM subtypes. Fruit Loop stimulus (Food) and ethanol stimulus (EtOH) were the two consumable food reward contexts used and female stimulus (FEM-Stim) and same-sex cage-mate stimulus (CM-Stim) were the two social contexts used. The percent of total calls for flat, trill, and non-trill FM subtypes observed for each rat were measured. Bars for each group across subtypes add up to 100%. Results are represented as means ± SEM. Presence of (**a**) indicates significant difference from control at $p < .05$. Presence of (**b**) indicates significant difference from cage-mate stimulus at $p < .01$. Presence of (**c**) indicates significant difference from food stimulus at $p < .05$. Presence of (**d**) indicates significant difference from both female and cage-mate stimulus at $p < .01$.*

Planned simple contrasts between each experimental group and the control on the average proportion of non-trill FM call subtypes indicated that both non-social groups (food and EtOH) had a significantly lower proportion of non-trill FM calls compared with control (both $p < .05$). Neither social group (female or cage-mate) significantly differed from the control in proportion of non-trill FM calls. Games-Howell post-hoc comparisons across the experimental groups found that the non-social conditions did not significantly differ from each other ($p = .816$), though the food group differed from both social groups (both $p < .01$) with a significantly lower proportion of non-trill FM calls observed. EtOH did not differ from either social condition (both $p > .05$). For social conditions, both, as stated, differed from food group, but did not differ from each other ($p = .910$; see Figure 3-5).

Discussion

The present study found that rats emit 50 kHz USVs at different rates and with different proportions of subtypes upon presentation of consumable or socially rewarding behavioural stimuli. The hypothesis that social stimuli would induce the strongest rate of 50 kHz USV emission was only partially supported. The simple procedure of exposing a naïve male rat to a naturally cycling female with no conditioning was sufficient to significantly increase 50 kHz USV emission across multiple sessions and in particular increase the proportion of trill calls emitted. This robust effect of a social context was not found using a reunion with cage-mate stimulus. The cage-mate stimulus failed to significantly increase 50 kHz calling or modulate subtype proportions.

The hypothesis that the flat 50 kHz USV call type would be associated with feeding behaviour was supported. Independent of the specific type of consumable food

reward (Fruit Loops or EtOH), we found experimental evidence of an association between food stimuli and the flat USV subtype. However, the hypothesis that this flat subtype would differentiate the social conditions as a 'social contact call' was not supported as the two social-stimulus experimental groups did not differ in the proportions of flat calls observed. The analysis of sonographic characteristics (peak frequency, duration, and bandwidth) of 50 kHz USVs found no significant effect of any behavioural context. However, 50 kHz USV calling in all behavioural contexts was susceptible to dopaminergic antagonism using haloperidol. Thus, we may conclude that all of the call subtypes in all conditions were dependent on mesolimbic dopamine.

In the current study, the only experimental stimulus to significantly induce 50 kHz USV emission was exposure to a female conspecific rat, and it did so across multiple recording sessions. The male rats in the female exposure group required no prior experience or conditioning to produce a high rate of 50 kHz calls as observed by call rates in the first exposure session. The procedure we employed allowed for visual, auditory, and olfactory cues between the two rats. Using a similar procedure, McGinnis and Vakulenko (2003) found that direct presence of a female stimulus rat without physical access was the most powerful inducer of 50 kHz calling, when compared with the conditions of either neutral bedding or bedding exposed to an estrus female. We extend these authors findings both by using a naturally cycling female stimulus and by finding evidence that this context is a more powerful inducer of 50 kHz calling than non-female social and non-social reward contexts. Though naïve rats produced a significantly higher 50 kHz call rate to the female upon first exposure than to baseline, the accumulation of experience appeared to reflect a sensitization of calling across recorded

sessions. The highest rate of calling was observed in the fourth recording session. This may appear paradoxical given that the USVs recorded were in the absence of the female (the male moved into the adjacent chamber after the female was removed). However, an increase in sexual arousal, even without possibility of mounting the female, has been found to possess a rewarding value (Zamble, Hadad, Mitchell, & Cutmore, 1985; Zamble, Mitchell, & Findlay, 1986). It is thus possible that this rewarding aspect of sexual arousal was driving the calling behaviour observed.

The lack of significant effect by the cage-mate, food, and EtOH stimuli to increase 50 kHz USV emission at rates greater than observed at baseline may result from an insufficient motivational state for the relevant context. Though these stimulus contexts have been previously shown to be capable of inducing 50 kHz USV calling (cage-mate: Willey & Spear, 2012; food: Burgdorf et al., 2000; EtOH: Buck et al., 2014a), animals are often separated, or food deprived for considerably longer periods of time. Both social isolation and food deprivation have been found to enhance 50 kHz calling in relation to relevant stimuli (Willey & Spear, 2013; Brenes & Schwarting, 2015). We attempted to employ minimal deprivation and conditioning standards for a more informative comparison with the female stimulus condition. It is thus reasonable to suppose that if the respective experimental groups had undergone greater food- or social-deprivation the stimuli would have been sufficient to increase 50 kHz calling.

Due to the need to prevent confounds across experimental groups, the testing apparatus did not have bedding present. This absence of bedding may have raised the threshold of induction for calling as it has been shown to be critical for calling (Natusch & Schwarting, 2010). Interestingly, 50 kHz call emission after 1.5 mg/kg AMPH

administration in the same apparatus without bedding was significantly attenuated (data not shown). Regardless, the relative comparison made across the experimental groups illustrates the difference in potency the female exposure stimulus has for 50 kHz USV production.

The results of directly comparing 50 kHz USV subtype proportions across four different reward-related contexts provide some measure of behavioural relevance to measured call types. We found evidence of an experimental induction of flat 50 kHz USVs relative to control through exposure to a consumable food reward (either food or EtOH). Associations between feeding behaviour and flat calls have predominantly been correlational in nature (Takahashi et al., 2010; Opiol et al., 2015). In the present study, we found evidence of a procedural induction of flat calls unique to consumable food contexts. Buck and colleagues (2014b) found an increase of both FM and flat USVs (though not trill) in a cue-paired food context. It is possible that these authors found an increase of FM calls in addition to flat as a result of the nature of the cue-paired paradigm they employed. The unpredicted cue paired with food may have induced anticipatory 50 kHz calls which Opiol et al. (2015) found were more likely to be FM.

Indeed, consistent with this explanation, we found an increased ratio of FM to flat USVs for both non-social experimental groups in the recording window prior to access to the consumable reward when all recording sessions were averaged (data not shown). The ratio of greater flat calls relative to FM calls occured only in the second recording window when the food was accessible. However, this anticipatory difference was not found in the first recording session and did not extend to subtype proportion differences between recording windows, and thus does not explain the present findings. In contrast to

our results and those of Buck et al. (2014b) the use of a food stimulus by Willey and Spear (2013) produced no significant effect on 50 kHz USV subtype. It is unclear to what degree this difference may result from differences in experimental procedure, analytic techniques, or strain differences.

The current findings failed to support the notion that flat 50 kHz USVs may have particular behavioural relevance in same-sex conspecific communication. This may have resulted from a lack of social motivation due to the short amount of isolation time used (cage partners only separated for 1.5 h), though Willey and Spear (2013) used a same-sex conspecific stimulus to induce 50 kHz calling and found no significant effect of social isolation on call subtypes emitted. These findings do appear to challenge the notion that flat 50 kHz USVs predominate in non-play social contexts. The present investigation found that there was a relatively greater proportion of FM calls compared to the non-social conditions which is consistent with the literature on conspecific play (Burgdorf et al., 2008; Kisko et al., 2017). Indeed, the anticipation of play with a juvenile partner was repeatedly found to elicit FM calls from male juvenile rats (Knutson et al., 1998; Burke et al., 2017). In the present work, the increased emission of FM calls by animals expecting their cage-mate but finding their absence may reflect a carryover of the emotional state established in anticipation. Thus, even though the USVs recorded were in the absence of the conspecific cage partner, the type of 50 kHz USVs that predominate may be suited to facilitate pro-social engagement. This explanation would appear to be consistent with the finding of a greater number of arm entries and distance travelled in a radial maze apparatus by same-sex conspecifics to frequency-modulated calls compared with 50 kHz

sine wave tones which controlled for all acoustic parameters beyond frequency modulation (Wöhr & Schwarting, 2007).

Beyond increasing the proportion of general FM USV types, the female stimulus specifically increased the trill subtype. The full implications of this finding are difficult to assess. It is plausible that the increase of trill calls reflects an intention to communicate specifically with the female. Presumably, this communication from the male rat could function to induce approach and facilitate copulation with the female. However, some playback studies appear to disagree about whether male 50 kHz USV emission fulfills this important function (Snoeren & Agmo, 2014; Willadsen et al., 2014). The approach behaviour towards 50 kHz calls by females in the absence of an olfactory cue or the presence of the male rapidly extinguishes, suggesting the approach behaviour by females results from the presence of the male being signaled rather than from the calls *per se* (Wöhr & Schwarting, 2012; Wöhr, 2018). Among same-sex conspecifics a 50 kHz sine wave tone designed to control for frequency-modulation was sufficient to induce approach behaviour (Wöhr & Schwarting, 2007). This may not, however, fully generalize to male-female dyads.

An alternative explanation, though not mutually exclusive, is that the trill call represents a specific level of frequency modulation reflective of high levels of positive emotional arousal. This is consistent with the finding of trill calls specifically increased after amphetamine administration (Wright et al., 2010; Mulvihill & Brudzynski, 2018) and during juvenile playful encounters (Burke et al., 2018). Additionally, this arousal explanation is consistent with our current finding of call induction and increased trill proportion occurring in the absence of the female conspecific. Importantly, this trill call

type and its induction by either female stimulus or amphetamine, is particularly susceptible to dopamine or noradrenergic receptor antagonism and unilateral depletion of dopamine with 6-hydroxydopamine (Ciucci et al., 2007; Wright, Dobosiewicz, & Clarke, 2012; Ringel, Basken, Grant, & Ciucci, 2013; Wright et al., 2013). Thus, trill calls may represent a high-arousal call and not be limited to a specific behavioural context, while alternatively, non-trill FM calls may serve some communicatory function as observed in juvenile play (Burke et al., 2018). Further research investigating the detailed mechanism underlying specific call types across a variety of behavioural contexts is needed.

Conclusion

In aggregate, the present data provide empirical evidence of behavioural relevance for both flat and FM 50 kHz USV subtypes in male Long Evans rats. Consumable food rewards appear to preferentially induce flat calls while social contexts elicit a greater proportion of FM calls. Moreover, a simple female-exposure stimulus was sufficient to induce high rates of 50 kHz USV emission across multiple recording sessions from naïve male rats, with a significant increase in the proportion of the trill subtype specifically observed. The use of a same-sex conspecific failed to elicit a greater proportion of flat calls. Beyond subtype proportion differences the distinct types of stimuli did not elicit qualitative alterations in the acoustic parameters of peak frequency, duration, or bandwidth. All USVs belonged to the same species-specific category. 50 kHz USVs emitted in each stimulus context were susceptible to dopamine antagonism using haloperidol and were, therefore, dependent on mesolimbic dopamine. Further investigation into the mechanisms underlying various non-pharmacological call-induction protocols is required.

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Rationale for Chapter 4

Emission of 50 kHz USVs has been demonstrated to be dependent on dopamine signaling and the activity of the ascending mesolimbic dopamine system. Disruptions to the function of the ascending mesolimbic dopamine system appear to block the capacity to emit 50 kHz USVs (Burgdorf et al., 2007; Ciucci et al., 2007, 2009). The results of chapter 3 were consistent with this dependence; results indicated that even 50 kHz USVs induced by non-pharmacological contexts could be attenuated by dopamine receptor antagonism. Most pharmacological research on inducing 50 kHz USVs has utilized psychostimulants administered either systemically or directly into the nucleus accumbens. This structure is extensively innervated by ascending dopamine fibers and represents the most well established forebrain area underlying 50 kHz USV emission. A central aim for chapter 4 was to establish the sufficiency of the neurotransmitter dopamine acting locally in the nucleus accumbens for inducing 50 kHz USV emission.

Additionally, a secondary aim of chapter 4 was to compare the 50 kHz USVs induced by dopamine with those induced by microinjections of amphetamine into the nucleus accumbens. Investigating this may shed light on whether the association of frequency modulation and call rate observed when amphetamine is used to induce 50 kHz USVs is unique to the pharmacological mechanisms of the drug.

Chapter 4: Effect of microinjections of dopamine into the nucleus accumbens shell on emission of 50 kHz USV: comparison with effects of D-amphetamine

This chapter has been adapted from the published article:

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Introduction

The behaviour of emitting USVs by *Rattus norvegicus* has been established as a reliable marker of underlying emotional states (Brudzynski, 2009, 2015; Wöhr & Schwarting, 2013; Barker, Simmons, & West, 2015). The two primary categories of USVs emitted by adult rats are the 22 kHz and 50 kHz USV types. These broad call categories are defined by both sonographic character (including call duration, sound frequency and bandwidth) and the different behavioural situations (and accompanied emotional states) associated with their emission (Brudzynski, 2009, 2013). There is abundant evidence which relates these 22 and 50 kHz call categories with two distinct neurochemical brain systems (for review see Brudzynski, Silkstone, & Mulvihill, 2018). The ascending mesolimbic cholinergic system is responsible for initiating the aversive emotional arousal expressed by 22 kHz USV emission (Brudzynski & Bihari, 1990; Brudzynski, 1994, 2001, 2010; Brudzynski & Barnabi, 1996). In contrast, the ascending mesolimbic dopaminergic (DAergic) system appears responsible for initiating the positive emotional arousal expressed by 50 kHz USV emission (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007; Ciucci et al., 2007, 2009; Brudzynski, 2009; Scardochio, Trujillo-Pisanty, Conover, Shizgal, & Clarke, 2015). This system is characterized by projections of dopamine (DA) fibers, from neurons located in the ventral midbrain (e.g., in the ventral tegmental area; VTA) to rostral brain regions associated with emotional processing (Alcaro, Huber, & Panksepp, 2007; Ikemoto, 2007). The most highly innervated rostral structures by ascending VTA DA fibers are the nucleus accumbens (NAc) and olfactory tubercle (OT), which together form the ventral striatum (Swanson,

1982; Voorn, Vanderschuren, Groenewegen, Robbins, & Pennartz, 2004; Ikemoto, 2007, Yetnikoff, Lavezzi, Reichard, & Zahm, 2014).

The emission of 50 kHz USVs occurs in a variety of positive appetitive contexts associated with the activity of this DA system, including both social and non-social rewarding situations (Knutson, Burgdorf, & Panksepp, 1998, 1999; Burgdorf et al., 2008; Brenes & Schwarting, 2015; Wöhr, 2018). For instance, Hori et al. (2013) found that 50 kHz USV emission in response to tickling (heterospecific play) was dependent on DA signaling within the NAc. Direct pharmacologically-induced release of DA at the terminal ends of these ascending mesolimbic pathways, such as via microinjections of amphetamine (AMPH) into the NAc, unconditionally elicited 50 kHz calling (Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Thompson, Leonard, & Brudzynski, 2006). Conversely, microinjections of AMPH into the caudate-putamen failed to influence rates of 50 kHz USV emission (Burgdorf et al., 2001). Beyond the use of AMPH, a range of agonistic DA agents have been linked with 50 kHz USV induction. The indirect agonist actions of systemic cocaine (Barker et al., 2010; Williams & Undieh, 2010, 2016), methylphenidate (Simola et al., 2012), and methamphetamine (Mahler et al., 2013), all similarly increased 50 kHz USV emission. All these drugs increase the concentration of DA within the synaptic cleft via altering the function of the plasma membrane transporter for DA (henceforth DAT; Zhu & Reith, 2008).

The dependence of 50 kHz USV emission on DA is further evidenced by studies disrupting the function of the ascending mesolimbic DA system. Depletion of DA levels in the rostral forebrain of animals by neurotoxic lesions of the ascending DA fibers greatly reduced the capacity of a rat to emit 50 kHz USVs to a previously rewarding

stimulus (Burgdorf et al., 2007; Ciucci et al., 2007, 2009; Grant, Barnett, Doll, Leverson, & Ciucci, 2015). Systemic (i.p.) antagonism of D_1-like and D_2-like DA receptors, both alone and in combination, significantly reduced 50 kHz USV production in response to an estrous female (Ringel, Basken, Grant, & Ciucci, 2013). Similarly, systemic administration of DA antagonists was found to attenuate 50 kHz USV emission induced by cocaine or amphetamine (Williams & Undieh, 2010, 2016; Scardochio & Clarke, 2013; Wright, Dobosiewicz, & Clarke, 2013). These findings indicate a necessary role of both D_1-like and D_2-like receptor subtypes for 50 kHz USV emission.

The use of non-psychostimulant drugs (including direct DA receptor agonists) to induce 50 kHz USV emission in a comparable manner to psychostimulants, however, has proved to be more complicated. Drugs that increase synaptic concentrations of DA (e.g., the DAT inhibitor GBR-12909) have failed to significantly induce 50 kHz USV emission in a manner comparable to AMPH when both were systemically applied (Wright, Gourdon, & Clarke, 2010; Wright et al., 2013). Systemic administration of the relatively non-selective D_1/D_2 agonist apomorphine was found by Williams and Undieh (2010) to significantly induce 50 kHz USV emission, while systemic administration of D_1 agonist SKF38393 or D_2 agonist quinpirole alone did not. Moreover, administration of direct receptor-selective agonists alone and in certain combinations was found to inhibit 50 kHz calling (Scardochio & Clarke, 2013). However, this avenue of pharmacological research is limited by the fact that systemic administration affects all aspects of the underlying neural circuitry associated with 50 kHz USV emission. This is especially important in consideration of the contributing presence of D_2-autoreceptors at both somatodendritic and axonal terminal areas (Ford, 2014). Within the shell of the NAc, direct administration

of quinpirole was found to induce 50 kHz USVs at a comparable rate to the response observed following 7 μg of AMPH (Brudzynski, Komadoski, & St. Pierre, 2012). This quinpirole-induced calling could be antagonized by either raclopride (D_2 antagonist) or U-99194A (D_3 antagonist), indicating contributions from both D_2 and D_3 receptors.

The various pharmacological agents used to induce 50 kHz USVs, including AMPH, or to antagonize specific DA receptors, appear to produce differences in the acoustic parameters of individual calls in addition to affecting the proportion of specific subtypes of USVs emitted (Wright et al., 2010, 2013; Brudzynski et al., 2012). It was found that AMPH selectively increased the proportion of trill calls (Wright et al., 2010), while the DAT blocker GBR-12909 was found to have failed to alter the 50 kHz USV profile (Wright et al., 2013). These differences may reflect the wide array of effects AMPH produces beyond increasing synaptic DA. AMPH is known to affect the noradrenergic and serotonergic systems as well as to inhibit the function of degradative enzymes (i.e., monoamine oxidase B, MAO; Sulzer, Sonders, Poulsen, & Galli, 2005; Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007). Both the noradrenergic and serotonergic systems have themselves been implicated in both the induction of 50 kHz USV emission and the alteration of call profile (Wright, Dobosiewicz, & Clarke, 2012; Wöhr, Rippberger, Schwarting, & van Gaalen, 2015).

Despite these off-target effects of AMPH, antagonism of D_1-like or D_2-like receptors was found to alter call profile and acoustic parameters of 50 kHz USVs, indicating differential contributions of receptor subtypes (Wright et al., 2013). Frequency-modulation of 50 kHz USVs appears particularly reliant on DA signaling, although, when drugs are given systemically it is difficult to rule out their effects on

cranial nerve control of vocal fold function (Ciucci et al., 2009; Ringel et al., 2013). General system-wide antagonism of DA receptors may disrupt the animal's ability to articulate frequency-modulated vocalization by altering laryngeal function (Feng, Henriquez, Walters, & Ludlow, 2009; Ringel et al., 2013).

The purpose of the present study was to investigate the capacity of using the native transmitter of DA as a pharmacological agent to induce 50 kHz USV emission when applied directly into the NAc. Given the complex landscape of off target actions that typical DAergic agents possess (particularly psychostimulants such as AMPH), using DA itself to increase the available concentration of transmitter capable of acting at post-synaptic receptors appears advantageous. Use of DA directly into the terminal field of the ascending mesolimbic DA system and comparing it with AMPH may reveal the character of 50 kHz USV emission primarily contributed by DA receptor action.

We hypothesized that microinjections of DA directly into the shell of the NAc would induce 50 kHz USV emission at a higher rate and with a decreased latency to call compared with vehicle. Additionally, the effects of DA on 50 kHz calling would differ as a function of increased dose and be susceptible to antagonism of D_2 receptors within the NAc shell. We further expected that the acoustic parameters of average call duration and bandwidth measured from individual 50 kHz USVs induced by DA microinjections would be greater than those following vehicle. It is this character of calling that appears most dependent on DA signaling (Ciucci et al., 2009; Ringel et al., 2013). In line with this, we expected DA microinjections to significantly increase the ratio of frequency-modulated USVs (FM calls) compared with flat calls. We did not expect that the acoustic character of 50 kHz USV calling or the ratio of FM calls would differ between DA and

AMPH microinjections on the assumption that the calls induced by AMPH result primarily from increased DA concentration in the synaptic cleft. To test these hypotheses and predictions, we employed direct microinjections of DA at varying doses into the shell of the NAc and compared results with those after AMPH with or without pre-treatment of D_2 antagonist raclopride, DAT antagonist GBR-12909, or vehicle on the emission of 50 kHz USVs.

Methods

Subjects

All 41 subjects were male Long Evans rats (obtained from Charles River Laboratories, Saint-Constant, QC, Canada). Subjects were given 5 days of acclimation time upon arrival into the animal facility. Before entry into the study, all animals were housed in pairs but following cannula implantation, they were housed singly. In accordance with Brock University protocols for laboratory handling, all animals were housed in polycarbonate cages (48 x 27 x 20 cm) with a plastic tube (polyvinyl) inside for hiding, an aspen block of wood for gnawing, and two paper towels for environmental enrichment. Cages were lined with dust-free corn cob bedding. The housing room had controlled room temperature (21° C ± 2° C) and humidity (40-60%). Subjects were housed with a maintained 12:12 h light/dark cycle and *ad libitum* access to standard rat chow (Harlan Laboratories, Wisconsin, USA) and water except while in the post-operative recovery period. During post-operative recovery from the stereotaxic surgical procedure, subjects were given access to wet chow and a purified dietary supplement (DietGel Boost®, ClearH20, Maine, USA). For the first day post-operation, housing cages were lined with paper-towels in place of bedding. All research protocols were

approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

Procedural overview

To investigate the possible dose-dependent effects of injected DA on 50 kHz USV emission, all animals received surgical implantation of guide cannulae through the skull into the brain. This cannulation allowed for intracerebral microinjection of drugs directly into the shell of the nucleus accumbens (NAcSh). A total of 25 rats were used for NAcSh injections with 15 subjects receiving injections of vehicle and three of six possible doses of DA (experiment 1; 3.75 µg, 7.5 µg, 15 µg, 30 µg, 60 µg, or 120 µg) and 10 subjects receiving a pre-treatment injection followed by either AMPH or DA into the NAcSh (experiment 2 and 3, five animals each). These latter experimental groups were used to better characterize the pharmacological response to DA microinjections and determine USV profile. Experiments 2 and 3 consisted of paired-injections (intracerebral or subcutaneous pre-treatment+ intracerebral microinjection treatment). Experiment 2 involved intracerebral injections of vehicle+dopamine (Veh+DA), raclopride+dopamine (Rac+DA), and vehicle+AMPH (Veh+AMPH). Experiment 3 involved subcutaneous pretreatment with GBR-12909 + intracerebral dopamine (GBR+DA) and subcutaneous pretreatment with vehicle + intracerebral AMPH (Veh+AMPH). GBR-12909 was used because it is a potent dopamine reuptake inhibitor (antagonist to the plasmolemmal DAT (Andersen, 1989). Systemic application of this DAT antagonist has been employed in investigations of USVs previously (Wright et al., 2013).

Recordings (10 min in duration) of USVs were carried out for all subjects immediately following any given microinjection. Number of calls and sonographic

parameters of individual calls were than analyzed for all recordings. The testing order for experiment 1 involved randomized order of all micro-injections (three DA doses as well as vehicle). In addition to this injection-order counterbalancing, each injection was separated by 7 days to provide a washout period and reduce the likelihood of sensitization. For experiment 2 the injection order for Rac+DA and Veh+DA was randomly counterbalanced across subjects, however, Veh+AMPH was the last injection for all subjects. This was done to prevent sensitization to AMPH from contaminating the other injections, although, again each injection was separated by 7 days. For experiment 3, a fixed injection schedule was employed with GBR+DA received first followed by Veh+AMPH after a 7 day washout period.

Stereotaxic surgeries

At the time of surgery, all subjects weighed approximately 300-350 g. All animals underwent stereotaxic surgery to receive implantations of stainless-steel guide cannulae (640 μm outer diameter, made from 23 gauge needles) bilaterally into the NAcSh. In preparation for the surgical procedure the rats were anesthetized with isoflurane (5% induction, 2% maintenance) and mounted on a stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, CA, USA). Upon achievement of appropriate anesthetic sleep depth, a dose of enrofloxacin antibiotic (enrofloxacin, Baytril®, Bayer DVM, at a dose of 5 mg/kg, s.c.) was administered to reduce risk of infection and a dose of analgesic (meloxicam, 2 mg/kg, s.c., Metacam, Boehringer Ingelheim Vetmedica, GmbH) was administered to reduce pain and discomfort upon waking. The surgical site was prepared by application of a 7% iodine scrub solution, followed by 70% isopropanol, and finally locally treated with 10% iodine.

Implantation coordinates for the NAcSh were 9.96 to 10.2 mm anterior to interaural line, 1.0 mm lateral from midline, and 6.0 to 7.0 mm ventral from brain surface according to coordinates from a stereotaxic atlas (Paxinos & Watson, 1986). Guide cannulae were secured to the skull using small stainless-steel screws and dental acrylic (DenPlus, Longueuil, QC) and were plugged with removable stainless-steel wires. No procedures took place on rats until five days post-surgery and only on healthy subjects; behavioural procedures (starting with habituation) began no later than 7 days post-surgery. Surgical procedures and post-operative care of the animals were always done with the supervision of an appointed veterinarian.

Drug injections and preparation

Stainless steel injection cannulae (30 gauge needles; 310 μm outer diameter, Beckton-Dickinson Canada, Mississauga, ON) connected to a Hamilton constant rate microsyringe (CR-700-20, Hamilton Company, Reno, NV) were used for intracerebral injections for all subjects. Injections were carried out at a rate of 0.2 μl/min with a volume of 0.3-0.5 μl injected at one side of the brain with hemisphere of injection counterbalanced across subjects. After injecting the given experimental substance, the injection cannula was left inside the guide cannula for at least 30 s to allow time for diffusion of the agent away from the cannula tip.

All drug solutions were prepared fresh the day of injection using sterile isotonic saline and were buffered to a pH of 5.5. Dopamine hydrochloride (Sigma-Aldrich Canada Ltd., Oakville, ON) was dissolved in varying concentrations for intracerebral application in several different doses (3.75 μg, 7.5 μg, 15 μg, 30 μg, 60 μg, or 120 μg) in 0.5 μl vehicle for animals in experiment 1. Vehicle and injectable solutions of DA contained

0.5% ascorbic acid as an antioxidant. Raclopride L-tartrate (Sigma-Aldrich, ON) was prepared for an intracerebral pre-treatment dose of 6.8 μg in 0.2 μl vehicle. *D*-amphetamine sulfate (dextroamphetamine, Sigma-Aldrich, Great Britain) was prepared for an intracerebral dose of 7 μg in 0.3 μl vehicle. GBR-12909 (Sigma-Aldrich, ON) was prepared for a systemic pre-treatment dose of 5 mg/kg s.c. in 0.5 ml vehicle. Each brain site was injected no more than four times.

The pre-treatment and treatment injections were separated by 10 min (with the animal in the home cage) and were given in a volume of 0.5 ml (s.c.) or 0.2-0.5 μl (intracerebral microinjection, see below) respectively. The dose of DA used for comparison with AMPH and raclopride microinjections into the NAcSh was 6 μg in 0.2 μl volume of vehicle. The dose of DA used for comparison with AMPH and GBR-12909 was 15 μg in 0.5 μl volume of vehicle. The dose of raclopride used for microinjections was 6.8 μg in 0.2 μl vehicle. The dose of AMPH for intracerebral microinjections was 7 μg in 0.3 μl vehicle. Doses were chosen for comparable molarity of microinjections across groups but also for half equimolar amount of raclopride relative to DA and near equimolar amount of DA and AMPH.

Histological procedure

At the end of the study, all animals were anesthetized by an overdose of barbiturate (sodium pentobarbital, Euthanyl, Vetoquinol N-A, Quebec, Canada). Brains were transcardially perfused and postfixed in a 10% formalin solution before being sectioned with a freezing microtome (Cryo-Histomat, Hacker Instruments and Industries, Fairfield, NJ). Histological sections (40-50 μm thick) were stained with thionine according to the original staining method by Windle et al., (1943), and looked at under a

light microscope to localize sites of injections. Localized sites were mapped onto coronal sections of the rat brain using a stereotaxic atlas (Paxinos & Watson, 1986). For a representative visual composite of localized injection sites see Appendix A.

Recording and analysis of ultrasonic vocalizations

All subjects received habituation to the injection and recording procedure for three days following recovery from the surgical procedure. This habituation involved gentle handling and exposure to the recording environment. The recording environment was dimly lit with a single table-top direct-current lamp. The recording procedure itself consisted of the subject being transported to the recording room in its home cage, the rat was taken out of the cage, then gently handled and given an intracerebral injection before being placed back into its home cage for 1 minute. The animal was then placed into a recording chamber (25 cm wide × 18 cm deep × 18 cm height polycarbonate cage) where USV production was recorded for 10 minutes. The recording chamber was filled with fresh corn cob bedding and was not re-used across rats; every subject received a new chamber for each recording.

All recordings were made using an UltraSoundGate CM16/CMPA (Avisoft Bioacoustics, Glienicke, Germany) condenser microphone (working frequency range 2-250 kHz) located on top of the recording apparatus on a metal grate (approximately 25 cm from the animal). The microphone was connected via an UltraSoundGate 416 USB audio device (Avisoft Bioacoustics) to a computer (Dell PC) and recordings were made using multi-channel triggering hard-disk software (Avisoft RECORDER version 4.40). Acoustic data were recorded at a sampling rate of 250 kHz in 16-bit format. Analysis of

USVs was done off-line using Avisoft SASLab Pro (version 4.40) and Sonotrack™ (Metris BV, The Netherlands) software (version 4.40).

USVs were analyzed and the identification and characterization of USVs was accomplished in a manner as described and used previously in several papers (Brudzynski, 2009, 2015; Mulvihill & Brudzynski, 2018a, 2018b). Briefly, 50 kHz USVs had peak frequencies between 35 and 90 kHz, were typically less than 100 ms in duration, and had varying degrees of frequency modulation. 22 kHz USVs were rare or absent but would be identified by having a low peak frequency (20 – 30 kHz), long call duration, and with constant frequency. Given the virtual absence of 22 kHz USVs this call type was omitted from the analysis. The two analysis programs were used for distinct and non-overlapping analyses in separate groups of animals. Reliability analysis for determining number of 50 kHz calls between the two programs indicated that performance of the Sonotrack progam is comparable to a competent experimenter performing manual detection using Avisoft program (Intraclass correlation coefficient = .945, 95% CI: .758, 988). Avisoft SASLab generated spectrograms were manually screened for 50 kHz USVs and were used to calculate sonographic parameters of peak frequency (in kHz), call duration (in ms), and bandwidth (in kHz) of individual calls for experiment 1 only. Additionally, Avisoft SASlab was used to determine numbers of 50 kHz USV subtypes, which were used to calculate the FM ratio for all experiments. Sonotrack generated spectrograms were utilized for preprogrammed automatic screening which determined number of USVs, mean call duration, and mean sound frequency of USVs for experiments 2 and 3 only. For automatic detection of 50 kHz calls by Sonotrack, a bandpass filter was employed to reduce background noise (low and high cut-

off frequencies of 35 and 90 kHz, respectively). All spectrograms were generated using a fast Fourier transform (512 FFT-length, 100% frame, Flat Top window, and 75% time window overlap), at 488 Hz of frequency resolution.

USV subtype determination was based on sonographic shape. 50 kHz calls were classified into the flat subtype if they appeared to have a relatively constant frequency (bandwidth < 6 kHz). If the calls were FM they were classified as either trill or non-trill subtypes. For calculating FM to flat call ratios all subtypes with frequency modulation (both trill and non-trill FM calls) were counted and divided by the number of flat 50 kHz USVs. Manual screening of 50 kHz USVs was accomplished by one trained experimenter.

Statistics

All statistical analyses were performed using SPSS Statistics (version 20, IBM Corporation). For experiment 1 comparisons, any variables with violations of the assumption of normality were corrected using nonlinear transformations. To accomplish this correction scores were all logarithmically transformed using the natural base of *e* following a linear transformation of +1. To initially establish an effect of DA independent of dose, the subject's DA injection scores were averaged and compared with vehicle using a paired t-test. For investigating the effect of doses, a repeated-measures ANOVA was used across DA injection dose-bins for the given within-subjects variable. DA dose-bins were constructed to account for the unequal n across each objective dose by converting the DA dose data into within-subjects variables. This was accomplished by categorizing injections within each individual subject into low, medium, or high relative doses. Given the low valid sample sizes for analyses involved in experiments 2 and 3

only non-parametric tests were employed. Wilcoxon signed-rank tests were used for comparisons between two paired variables in experiments 2 and 3.

A total of 41 animals were subjected to experimental manipulation (experiment 1: 27, experiment 2 and 3: 14), however, for analysis of USV call induction only subjects with valid injection site localization were used (16 subjects excluded). For any analysis on parameters of individual calls any non-callers were excluded in addition to these localization invalid subjects. The associated n of any analysis is reported with its respective figure, where appropriate.

Where suitable, bias-corrected and accelerated bootstrap (BCa) confidence intervals are reported utilizing 1000 bootstrap samples.

Results

Induction of 50 kHz USV emission by intracerebral DA microinjections

Collapsed across doses and compared with vehicle for experiment 1, there was evidence that DA induced 50 kHz call emission ($t_{(11)} = 2.55$, $p = .027$, BCa 95% CI [.200, 1.170]). The recorded response to DA ($M = 6.65$, $SD = 8.11$ USVs/min, range (difference between highest and lowest) = 28.1) had significantly elevated USV emission relative to vehicle ($M = 4.55$, $SD = 7.21$ USVs/min, range = 17.8; see Figure 4-1a). Call rate in the two conditions was found to be significantly correlated ($r = .70$, $p = .011$). This effect of DA compared with vehicle was not found in subjects where the injection site was localized outside of the NAcSh ($t_{(7)} = 1.87$, $p = .104$, BCa 95% CI [-1.29, -0.018]). To investigate any difference in call rate across doses of DA a repeated measures ANOVA was conducted on calls calculated as a percent change of vehicle across dose bins (low, medium, and high). No significant difference was found in call emission as a percent

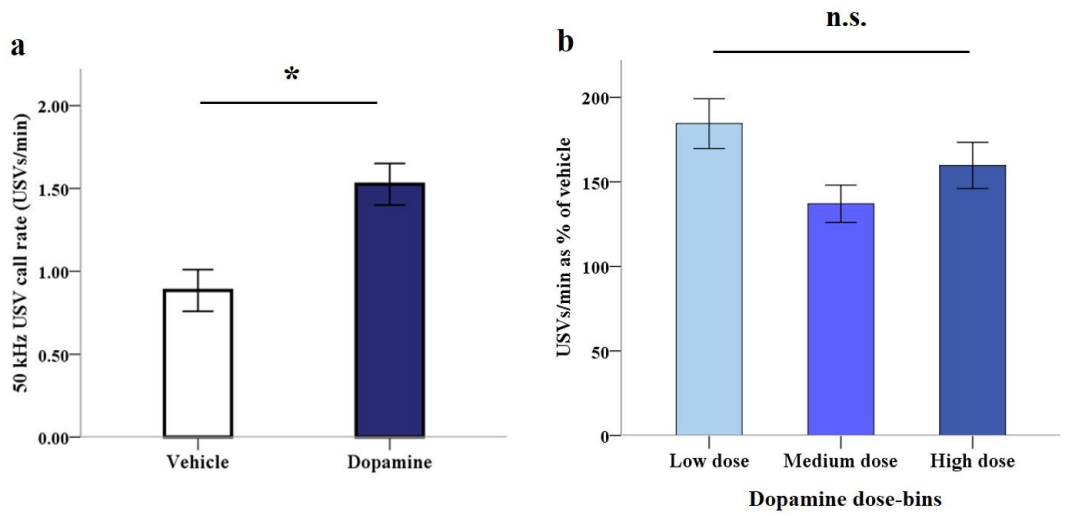

*Figure 4-1. (**a**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min between vehicle and dopamine microinjections (n = 12) for experiment 1. Dopamine call rate represents average call rate collapsed across dose. Dopamine induced significantly higher call rate compared with vehicle at p < .05. Results are represented as means ± SEM following logarithmic transformation using the natural base of e. (**b**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min across dopamine dose-bins as a function of percent of vehicle (n = 11) for experiment 1. No significant effect of dose-bin was found, although low dose trended towards statistical difference from medium dose. Results are represented as means ± SEM as percent of vehicle following logarithmic transformation using the natural base of e. * = p < .05. Use of (**n.s.**) denotes no significant differences across dose bins (at p > .05).*

change of vehicle across these dose bins ($F_{(2, 20)} = 2.14$, $p = .143$), although the difference between low and medium doses trended towards significance ($F_{(1, 10)} = 4.59$, $p = .058$; see Figure 4-1b).

Additionally, there was an effect of DA (collapsed across dose) to decrease latency to call (measured in seconds) compared with vehicle ($t_{(7)} = 2.55$, $p = .038$, BCa 95% CI [10.50, 57.12]). On average across DA recordings, there was a latency of 22 s (SD = 19.43) to emit 50 kHz USVs which was significantly shorter than the average latency of 55 s (SD = 47.54) observed after vehicle (see Figure 4-2). There was no significant difference found in latency to emit calls among particular doses of DA ($F_{(2, 14)} = 2.14$, $p = .155$).

Effect of intracerebral DA microinjections on acoustic parameters of individual calls

No significant difference was found in measured acoustic parameters (call duration, peak frequency, and bandwidth) between DA-induced USVs (collapsed across dose) and calls after vehicle microinjection recordings ($F_{(1, 7)} = 0.58$, $p = .472$; see Figure 4-3). There was also no evidence of any significant difference across doses of DA on average call duration ($F_{(2, 14)} = 0.89$, $p = .432$) or peak frequency ($F_{(2, 14)} = 1.78$, $p = .204$). For the average bandwidth of recorded calls however, there was a significant difference found across DA doses ($F_{(2, 14)} = 4.10$, $p = .040$). Pairwise comparisons found this difference resulted from the average bandwidth of calls recorded in low-dose conditions being significantly higher than those recorded under medium dose conditions ($p = .030$), but not under high dose conditions (see Figure 4-4). An analysis of the relationship between 50 kHz call rate induced by DA and average bandwidth induced by DA found a

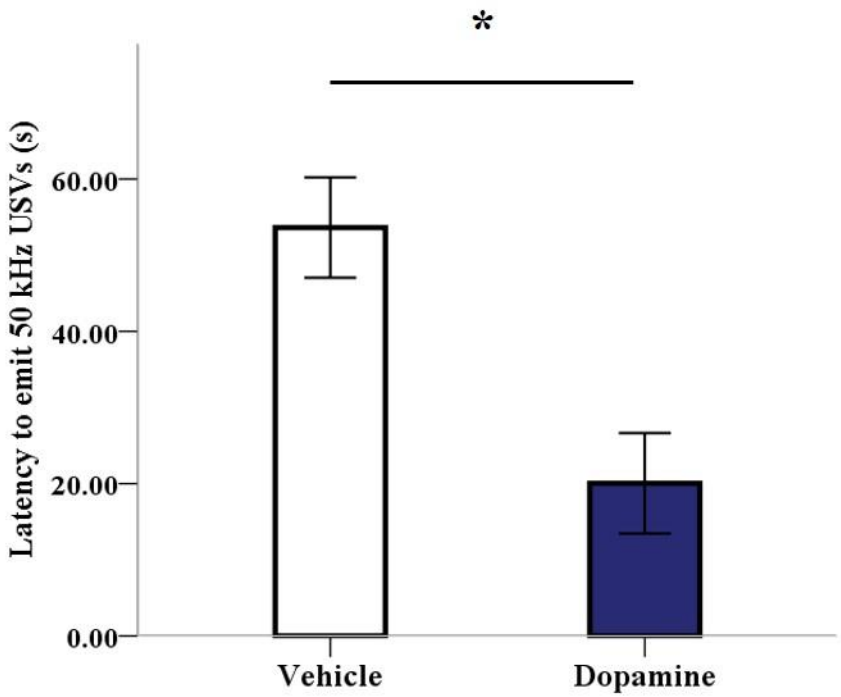

*Figure 4-2. Comparison of latency to emit 50 kHz USVs measured in seconds between vehicle and dopamine microinjections (n = 8) for experiment 1. Dopamine data represents average latency collapsed across doses. Dopamine microinjection recordings had a significantly lower latency to emit calls. Results are represented as means ± SEM. * = p < .05.*

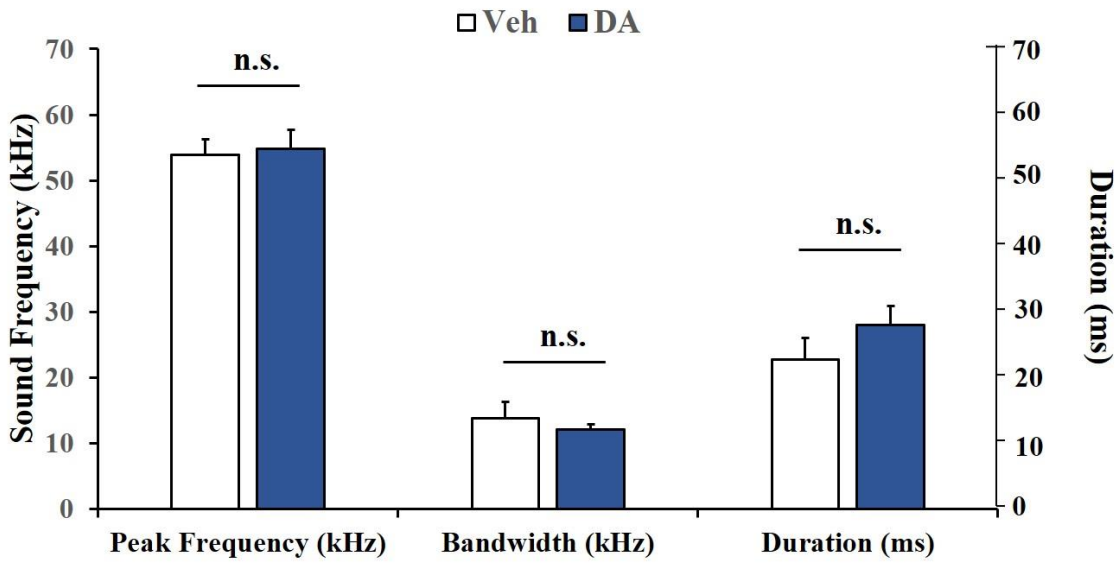

Figure 4-3. Comparison of acoustic parameters of individual 50 kHz USVs between vehicle (Veh) and dopamine (DA) microinjections (n = 8) for experiment 1. Dopamine data represents averages collapsed across doses. Peak frequency in kHz, bandwidth in kHz, and duration in milliseconds were measured for individual single calls. Bandwidth is expressed on the same axis as peak frequency in kHz. No significant differences were found between injection conditions for all acoustic parameters measured. Results are represented as means ± SEM. Other abbreviations as in Figure 4-1.

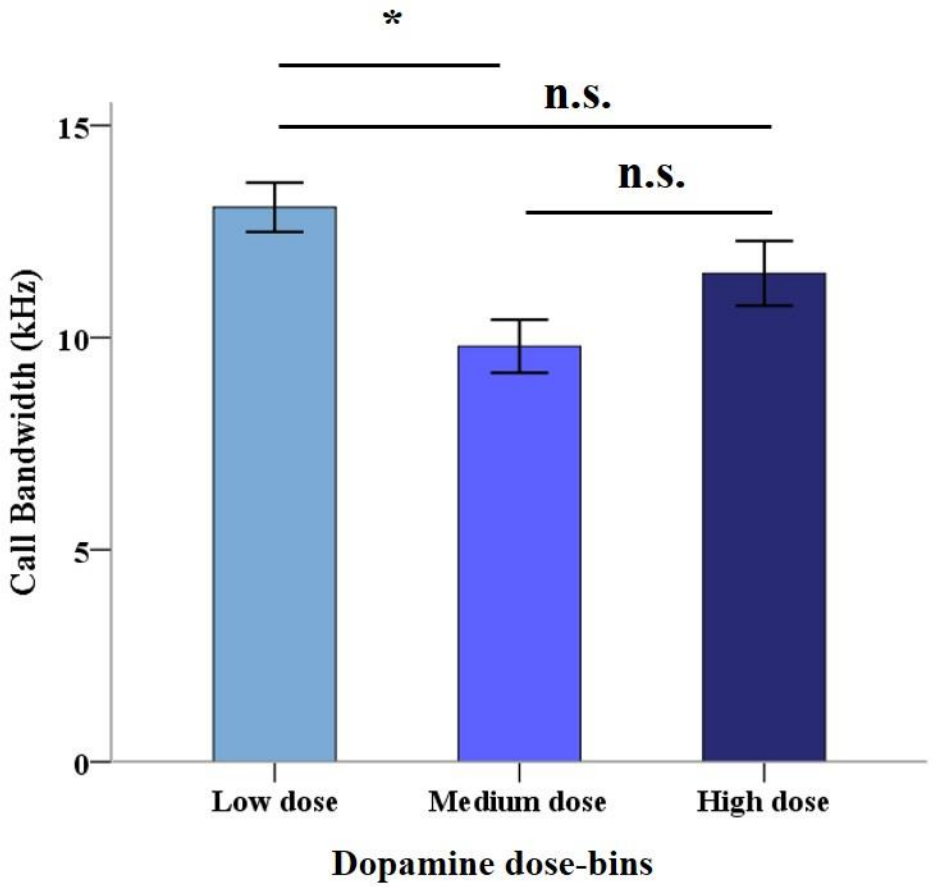

Figure 4-4. Comparison of average bandwidth of individual 50 kHz USVs across dopamine dose-bins (n = 8) for experiment 1. Bandwidth (measured in kHz) was found to be significantly higher under low dopamine dose conditions when compared with medium doses at $p < .05$. Results are represented as means ± SEM.

significant positive correlation (r = .613, p = .026). Individual rats that produced more USVs in response to DA microinjections appeared to have a greater likelihood of frequency modulation among individual calls (see Figure 4-5).

Effect of intracerebral DA microinjections on 50 kHz USV FM/flat ratio

Consistent with the finding of a positive correlation between DA-induced call rate and bandwidth, there was evidence found that DA altered the ratio of FM over flat 50 kHz USVs (FM/flat) when collapsed across dose and compared with FM/flat ratio after vehicle ($t_{(7)}$ = 2.40, p = .047, BCa 95% CI [.186, 1.009], see Figure 4-6). Recordings following DA microinjections had a greater average FM/flat USV ratio (M = 2.57, SD = 1.28) relative to vehicle (M = 1.96, SD = 1.39). No significant difference was found for FM/flat ratio among particular doses of DA ($F_{(2, 14)}$ = 0.87, p = .440).

Comparison of intracerebral DA microinjections with AMPH and GBR-12909

In a separate group of animals (experiment 2, n = 5), microinjections of vehicle into the NAcSh followed by AMPH (Veh+AMPH) were found to increase 50 kHz USV call rate compared to vehicle followed by DA (Veh+DA; *Medians* of 1.1 and 0.2 USVs/min, ranges of 8.4 and 0.8 respectively; see Figure 4-7a). This median difference was found to be statistically significant (Wilcoxon signed-ranks test, Z = 2.03, p = .042, r = .91). In addition to increasing call rate, Veh+AMPH increased average duration of individual USVs (Z = 2.02, p = .043, r = .91; see Figure 4-7b), though had no detectable effect on average sound frequency of individual USVs compared with Veh+DA condition (Z = 0.67, p = .500; see Figure 4-7c). In contrast, intracerebral pre-treatment with the D_2 receptor antagonist raclopride (6.8 μg in 0.2 μl vehicle) prior to DA, produced no difference in median call rate (Z = 1.07, p = .285), average call duration (Z = 1.83, p =

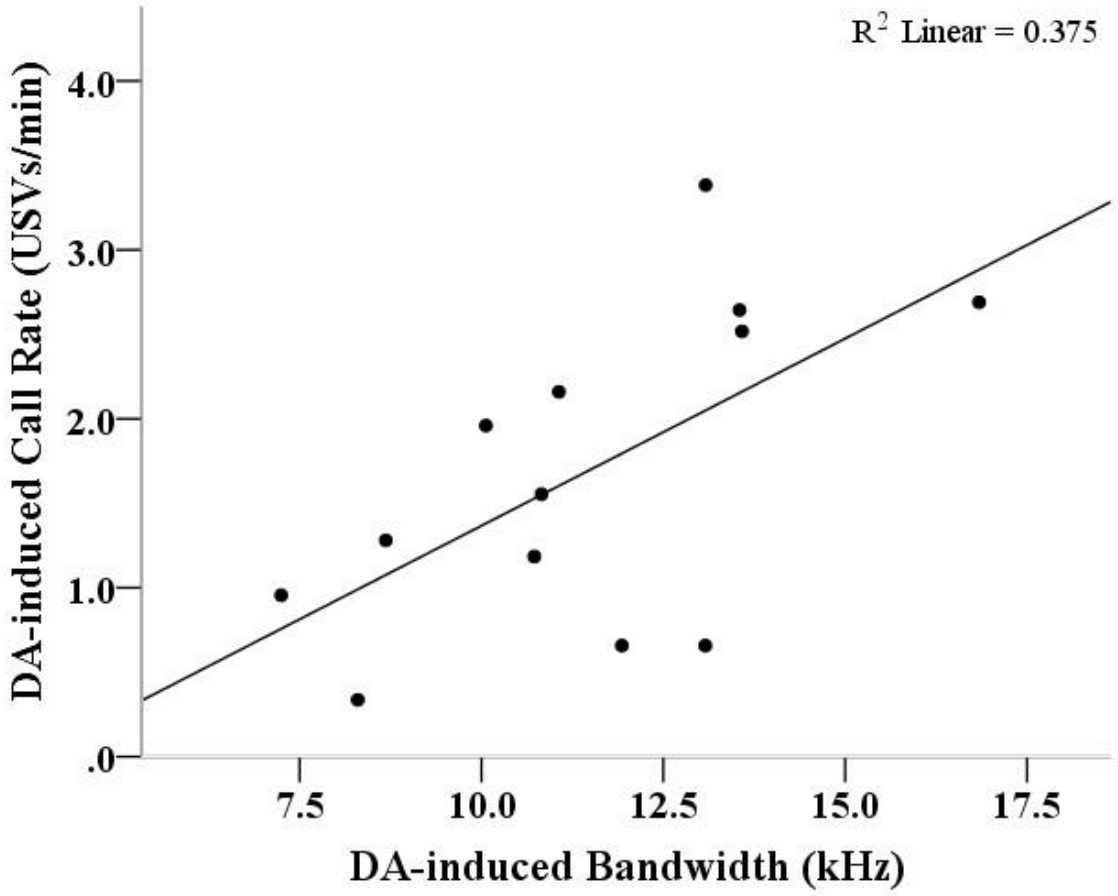

Figure 4-5. Scatterplot illustrating the positive relationship between log-transformed 50 kHz USV call rate induced by dopamine and average bandwidth of individual 50 kHz calls induced by dopamine (n = 13) in experiment 1. Dopamine induced call rate represents average call rate collapsed across dose following logarithmic transformation using the natural base of e. Dopamine induced bandwidth represents average bandwidth collapsed across doses in kHz. Correlation coefficient was found to be significant at p < .05.

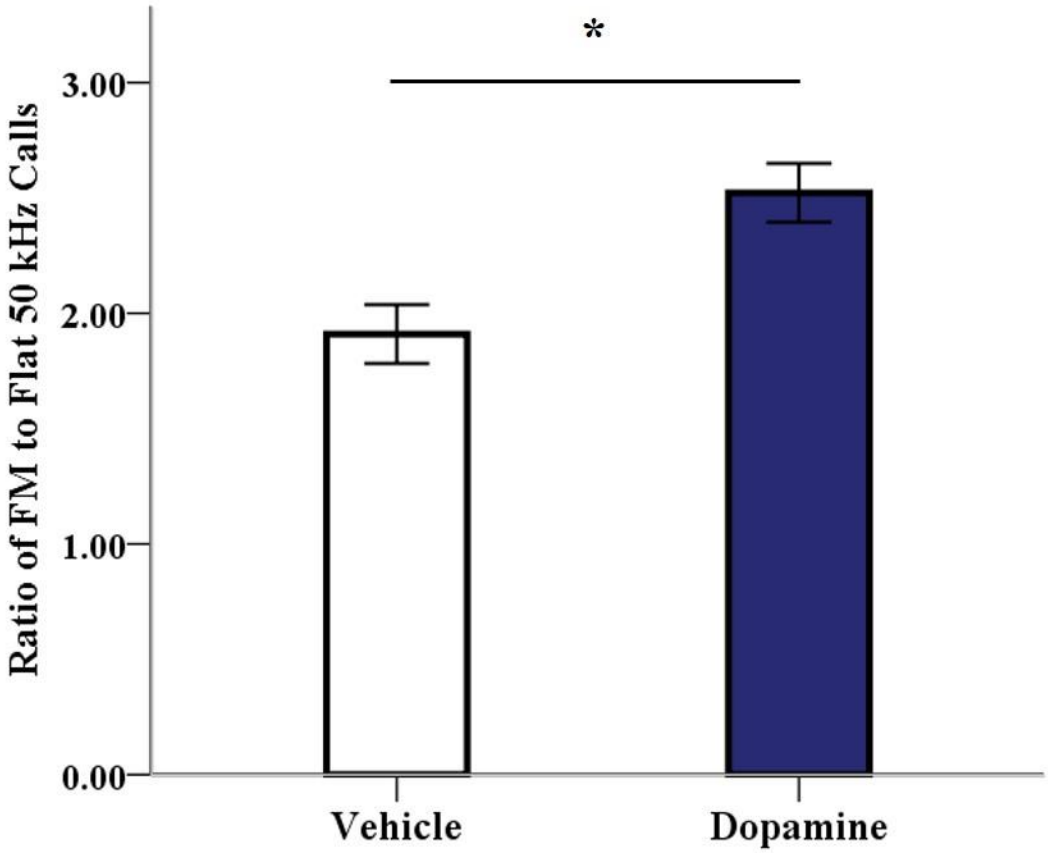

*Figure 4-6. Comparison of the ratio of frequency modulated (FM) to flat 50 kHz USVs recorded after vehicle and dopamine microinjections (n = 8) in experiment 1. Dopamine data represents average FM to flat ratio collapsed across doses. Dopamine microinjection recordings had a significantly higher ratio of FM to flat 50 kHz calls compared with vehicle. Results are represented as means ± SEM. * = p < .05.*

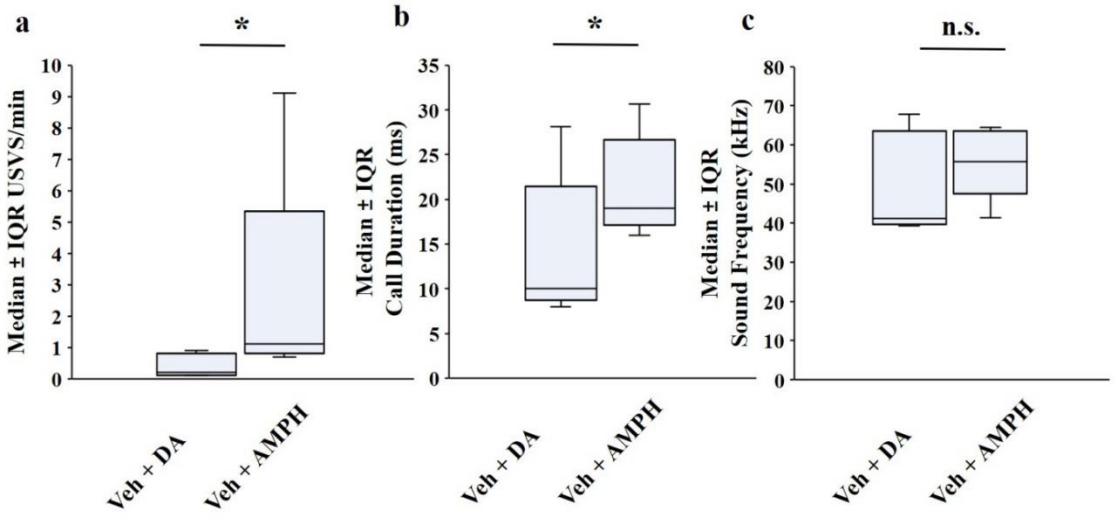

*Figure 4-7. (**a**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min after intracerebral vehicle pre-treatment with dopamine microinjections (Veh + DA) and intracerebral vehicle pre-treatment with amphetamine microinjections (Veh +AMPH) in experiment 2. (**b**) Comparison of average call duration (measured in ms) of individual 50 kHz USVs recorded after Veh + DA or Veh + AMPH microinjections in experiment 2. (**c**) Comparison of average sound frequency (measured in kHz) of individual 50 kHz USVs recorded after Veh + DA or Veh + AMPH microinjections in experiment 2. For all panels results are represented as medians ± the interquartile range (IQR) with n of 5. ***** - represents significant results of a related samples Wilcoxon signed-rank test at $p < .05$ level. Use of (**n.s.**) denotes no significant difference.*

.068) or sound frequency ($Z = 0.36$, $p = .715$) of individual calls compared with Veh+DA condition.

In an additional group of animals (experiment 3, n = 5), blocking DA re-uptake via systemic pre-treatment with GBR-12909 (GBR+DA, 5 mg/kg s.c.+ 15 μg/0.5 μl intracerebrally) appeared to mitigate the difference observed between DA and AMPH induction of USVs. The 50 kHz USV call rate following GBR+DA (*Median* = 1.9 USVs/min, range = 2.5) was lower than call rate after AMPH microinjections following s.c. vehicle pre-treatment (*Median* = 2.8 USVs/min, range = 8.9). This difference however, although trending towards it, did not reach statistical significance ($Z = 1.83$, $p = .068$; see Figure 4-8a). There was also no significant difference found in latency to call (measured in s) between these injection conditions ($Z = .73$, $p = .465$).

GBR+DA was also not found to significantly differ in average duration ($Z = 0.94$, $p = .345$, Figure 4-8b) or sound frequency ($Z = 1.21$, $p = .225$, Figure 4-8c) of individual 50 kHz USVs when compared with calls after systemic vehicle paired with microinjections of AMPH into the NAcSh. Investigating the ratio of FM relative to flat 50 kHz USVs between GBR+DA and AMPH induction found a significant difference ($Z = 2.02$, $p = .043$). Microinjections of AMPH into the NAcSh appeared to induce a greater number of FM calls relative to flat when compared with DA microinjections following GBR-12909 pretreatment (see Figure 4-8d).

Discussion

The purpose of this study was to explore the characteristics of 50 kHz USVs induced directly by DA microinjections into the shell of the NAc. The results support the hypothesis that DA microinjections (collapsed across doses ranging from 3.75 μg to 120

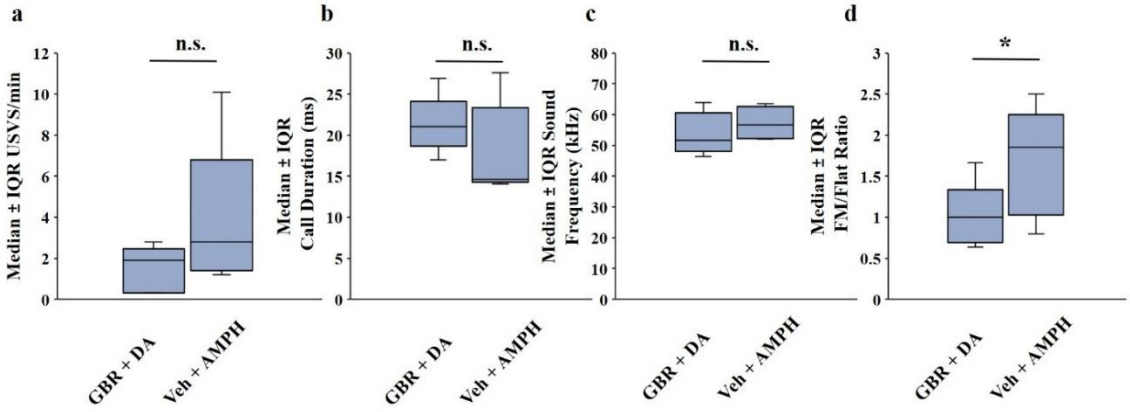

*Figure 4-8. (**a**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min after systemic GBR-12909 pre-treatment with dopamine microinjections (GBR + DA) and systemic vehicle pre-treatment with amphetamine microinjections (Veh +AMPH) in experiment 3. (**b**) Comparison of average call duration (measured in ms) of individual 50 kHz USVs recorded after GBR + DA and Veh + AMPH in experiment 3. (**c**) Comparison of average sound frequency (measured in kHz) of individual 50 kHz USVs recorded after GBR + DA and Veh + AMPH in experiment 3. (**d**) Comparison of the ratio of frequency modulated (FM) to flat 50 kHz USVs recorded after GBR + DA and Veh + AMPH in experiment 3. Results are represented as medians ± the interquartile range (IQR) with n of 5. ***** - represents significant results of a related samples Wilcoxon signed-rank test at p < .05 level. Use of (**n.s.**) denotes no significant difference.*

μg) can induce higher rates of 50 kHz USV emission with decreased latency as compared to calls after vehicle. However, we failed to find support for any differences in 50 kHz USV call rate induced across the individual doses used. Moreover, there was no evidence found that DA microinjections altered the acoustic parameters (duration, peak frequency, and bandwidth) of individual calls compared with those after vehicle. Unexpectedly, comparison among results of DA injections revealed differences in the average bandwidth of calls with the lowest doses producing the highest average bandwidth recorded. There was a significant positive relationship found between call rate induced by DA and the magnitude of average bandwidth of individual calls induced by DA. This finding was consistent with the hypothesis of a link between DA and frequency modulation.

Support for the hypothesis that direct intracerebral application of DA would affect the degree of frequency modulation observed among 50 kHz USVs was further found in comparisons of the ratio of FM to flat calls between recordings after DA or vehicle. Results found that calls recorded following DA injections had a higher ratio of FM calls relative to vehicle, which is in line with literature reports employing DA receptor agonists and antagonists (Wright et al., 2013).

In contrast with expectations, comparing results of DA and AMPH microinjections found that 7 μg AMPH was more effective at inducing 50 kHz USVs than DA and did appear to alter acoustic parameters of recorded calls. AMPH injections increased the average duration of individual calls compared with those after 15 μg DA though no difference was found for average sound frequency of calls. In contrast to several studies using psychostimulant USV induction (Thompson et al., 2006; Williams

& Undieh, 2010; Wright et al., 2013), the hypothesis that raclopride antagonism of D_2 receptors prior to DA administration would affect the character of calling was not supported. Raclopride pretreatment was not found to alter the measured acoustic parameters of individual calls induced by DA when compared with vehicle pretreatment.

Employing a systemic application of the DAT antagonist (GBR-12909) prior to DA microinjections was found to attenuate the differences in 50 kHz USV emission between DA and AMPH injections. Call rate induced by DA following GBR-12909 pretreatment was not significantly different from that after AMPH (though trended towards it). Given the lack of a control for the effect of GBR 12909 alone, we are unfortunately limited in our ability to comment on the specific contribution of dopamine within this comparison. The acoustic parameters of average duration and average sound frequency were also no longer different between DA and AMPH-induced 50 kHz USVs following GBR-12909 pretreatment. However, AMPH was found to induce a greater ratio of FM to flat calls compared with the ratio after DA following pretreatment with a transporter antagonist. Thus, the hypothesis that there would be no difference in the ratio of FM calls between AMPH and DA induced 50 kHz USVs was not supported. This dissimilar finding between ratio of frequency modulated calls and acoustic parameters may suggest differential sensitivity among these measures. AMPH has been found to preferentially increase the trill call subtype (Wright et al., 2010) and this appears consistent with our finding of increased FM calls relative to flat. One possible explanation as to why this increase in FM calls was not reflected in analysis of acoustic parameters may be due to the sonographic nature of the increased FM calls. Trill calls, which may have driven the difference, depend on peak-frequency modulation and our

measure of average sound frequency may not have reflected this sonographic difference (Pereira, Andreatini, Schwarting, & Brenes, 2014).

In aggregate, the results of this study support the hypothesis that drugs capable of inducing 50 kHz USVs via increasing synaptic DA concentration (i.e. AMPH) reflect primarily the influence of DA within the NAcSh on 50 kHz call profile and acoustic parameters. Furthermore, the apparent relation of frequency modulation of 50 kHz USVs and DA found in studies employing invasive disruptions or exogenous ligands to alter the function of the mesolimbic DA system was supported using the native transmitter. To our knowledge, this study represents the first use of DA as a pharmacological agent to induce emission of 50 kHz USVs from the rat.

The lack of a difference in 50 kHz USV emission rate across DA doses found in the present study was unexpected. This finding likely informs that the doses and local microinjection procedure used in the current study were insufficient to overcome brain mechanisms of DA elimination. Prior work employing intracerebral DA microinjections to explore its behavioural effects in rats utilized a MAO inhibitor in order to prolong the synaptic action of DA (Pijnenburg & Van Rossum, 1973; Costall & Naylor, 1975; Jackson, Andén, & Dahlström, 1975; Pijnenburg, Honig, & Van Rossum, 1975). In the current work we did not employ any such inhibitor as this would limit the value of comparing the effect of DA injections with that of AMPH injections, because as mentioned, one of the effects of AMPH is to inhibit the functional activity of MAO (Sulzer et al., 2005). Instead, we used an antagonist of the DA plasma membrane transporter (GBR-12909) to prolong the synaptic action of the injected DA. This pretreatment was found to show a trend of increasing the number of 50 kHz USVs

induced by DA and abolished many of the differences that were found between DA and AMPH following only a vehicle pretreatment.

Even without MAO inhibition, Pijnenburg et al. (1976) found a stimulatory effect on locomotor activity with a dose of DA as low as 5 μg injected directly into the NAc. In the present work, we were not able to obtain locomotor activity data coincident with USV recordings which would allow for a direct comparison. Also, the dose range for induction of USVs might be different from that for locomotor activity. Changes in rat behavior after intraaccumbens injection of DA seem to be inducible by a wide range of doses and behavioural changes were reported after doses as high as 200 μg (Costal & Naylor, 1975).

Microinjections of AMPH into the NAcSh were found to be more efficacious than DA in inducing emission of 50 kHz USVs as well as altering the call profile in favour of frequency modulation. DA itself increased the ratio of FM to flat calls compared with vehicle, but AMPH appeared to increase this ratio when compared with DA following transporter inhibition. This effect of AMPH-increased frequency modulation is abundant in the literature, including evidence of sensitization of FM USVs which itself is different from locomotor activity sensitization (Wright et al., 2010; Taracha et al., 2014; Simola & Morelli, 2015). It is possible that this finding of AMPH-increased FM ratio compared to DA reflects a difference in the length of time for DA signaling in the synaptic cleft between the two conditions. In both conditions the clearance rate of transmitter is largely dependent on the enzymatic activity of catechol-o-methyltransferase (COMT). However, under AMPH conditions, the drug induces efflux of DA molecules from the cytoplasm and also interrupts the vesicular transporter cycle; thus, it may be capable of saturating

this enzyme for longer periods of time (Fleckenstein et al., 2007; Sitte & Freissmuth, 2015). In accordance with this reasoning, the microinjections of dopamine used in this study (with their putatively fast transient duration) may have acted in a similar fashion as phasic dopamine release within the NAc. Such phasic dopamine release has been found to be associated with both the production and reception of 50 kHz USVs (Willuhn et al., 2014; Scardochio et al., 2015). The investigation of the role of this phasic dopamine release indicates that it may be capable of inducing 50 kHz calling but does not appear sufficient for maintenance of the behaviour (Scardochio et al., 2015). Delineating the association of local AMPH effects and tonic/phasic DA signaling in 50 kHz call induction may represent an exciting avenue for future research.

An alternative explanation for the difference in FM ratio induction between AMPH and DA observed in the present work relates to the general catecholaminergic effects of AMPH. In addition to its DAergic effects, the noradrenergic effects of the drug may explain its greater capacity to increase frequency modulation (for relevant review see Rippberger, van Gaalen, Schwarting, Wöhr, 2015). Wright et al. (2012) found that systemic α_1 receptor antagonism (prazosin) or α_2 autoreceptor agonism (clonidine) dose-dependently reduced AMPH-induced 50 kHz USV emission. Importantly, the α_1 antagonism prevented the typical alteration in call profile of increased FM calls. Similar application of a β_1/β_2 receptor antagonist (propranolol) showed no effect on AMPH-induced 50 kHz USV call rate but did dose-dependently alter the call profile with an increased proportion of flat calls at the expense of the proportion of FM calls. These findings may be instructive as to how AMPH affects 50 kHz USVs; however, caution

must be exerted in generalizing findings from systemic application of drugs to the current work involving local intracerebral NAc application.

There are several noted limitations specific to this study. Following exclusions due to cannulae localization, non-calling, or missing data across injection conditions, the effective sample sizes for any given statistical analysis were low. Thus, the findings of the present work should be considered exploratory in nature. Moreover, in part due to this low sample size, the comparisons between DA and AMPH microinjections required the use of non-parametric statistical tests which may have failed to detect differences between certain variables. The results also may not generalize beyond the doses used in the present study or subjects of different age, sex, or strain. Further research is required to perform an exhaustive comparison between DA- and psychostimulant-induced 50 kHz USV emission. Future studies may investigate the contributions of different locations within the brain to establishing the species-typical 50 kHz USV call profile inducible by microinjections of DA.

The two software programs used in the current study (Avisoft SASlab Pro and Sonotrack™ by Metris), although used for distinct purposes, were found to be generally complementary to each other. The automatic detection and screening for 50 kHz calls among spectrograms using Sonotrack enabled a much greater breadth of recordings to be analyzed. However, the manual precision afforded by Avisoft SASlab Pro was critical in determining the holistic sonographic architecture of recorded 50 kHz USVs (i.e. call subtypes).

Conclusion

Microinjections of DA into the NAc in the rat were demonstrated to increase call rate and decrease latency to call relative to vehicle. To our knowledge, this is the first study to employ intracerebral microinjections of DA into the brain to induce 50 kHz USVs. DA microinjections into the shell of the NAc were not found to differ from vehicle in any of the measured acoustic parameters for individual calls (peak sound frequency, duration, or bandwidth). However, calculated as a ratio of FM to flat calls, it was found that DA increased the ratio of FM calls compared with vehicle. Antagonism of the D_2 receptor using intracerebral raclopride was not found to inhibit DA-induced 50 kHz calling. Microinjections of AMPH into the NAc shell was found to be more effective than DA given alone (in the dose-range used) for increasing call rate and average duration of calls. Blocking the DA reuptake transporter successfully minimized these DA-AMPH differences in call rate and average duration of calls; however, AMPH was found to have increased the ratio of FM/flat calls. Together these results suggest that DA signaling even locally injected into the shell of the NAc is capable of inducing 50 kHz USV emission. Moreover, a number of psychostimulants may differ in the character of 50 kHz USV emission as a function of their pharmacological profile compared with the native transmitter acting in isolation. This consideration may be important when extracting meaningful contributions of singular brain neurochemical systems via application of pharmacological agents to induce organismal behaviour.

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Rationale for Chapter 5

Behavioural sensitization of locomotor activity and emission of 50 kHz USVs after repeated amphetamine injections in rats has been demonstrated repeatedly. These two behaviours appear dissociable in their sensitization patterns (Taracha et al., 2012, 2014; Garcia & Cain, 2016). Both behaviours in the rat are thought to be reflective of the underlying emotional and arousal states of the organism and may be robustly induced by psychostimulant administration. A central aim of chapter 5 was to determine if the evidence of dissociation between 50 kHz USVs and locomotor behaviour could be extended to a more general and sensitive measure of ergometric motor activity in a minimal sensitization protocol.

Additionally, although amphetamine is often used to induce 50 kHz USVs, there is little research to date on the extent of possible cortical and subcortical forebrain region involvement in 50 kHz call production after the drug. My research in chapter 5 was aimed at using patterns of expression of the inducible transcription factor Zif following a minimally sensitized amphetamine injection to explore the possible involvement of various forebrain regions in 50 kHz USV emission. In particular, medial prefrontal and striatal regions were investigated as these regions align most closely with the putative anatomical subcomponent of the ascending mesolimbic dopamine system responsible for establishing positive emotional arousal. Evidence of activity in these regions being associated with emission of 50 kHz USVs observed following AMPH would support the idea that the forebrain regions underlying this emotional expression extend beyond the shell of the nucleus accumbens.

Chapter 5: Investigation of 50 kHz USVs and ergometric activity with systemic amphetamine and Zif-268 immunohistochemistry.

Author contribution: For this manuscript I conducted all aspects of the ethics approval process and experimental work. I carried out all writing and figure construction by myself with feedback and edits provided by Dr. Stefan Brudzynski.

Introduction

The measurement of ultrasonic vocalizations (USVs) in adult rats is thought to reflect internal motivational and emotional states (Brudzynski, 2009, 2013). Recording of rat USVs has been utilized effectively as a quantifiable metric of emotional states in a wide variety of experimental settings (Wöhr & Schwarting, 2013; Rippberger, van Gaalen, Schwarting, & Wöhr, 2015). The two primary USV categories (the 22 kHz and 50 kHz USV types) in the rat are differentiated by general sonographic character (occupied sound frequency, call duration, etc.), the context their emission occurs in (negative versus positive valence contexts), and their underlying neurochemical systems (for reviews see Brudzynski, 2015; Brudzynski, Silkstone, & Mulvihill, 2018). Emission of 50 kHz USVs is generally associated with activity of the ascending mesolimbic dopamine system as both direct and indirect modulations to the activity of this system produce profound effects on calling behaviour (Knutson, Burgdorf, & Panksepp, 1998, 1999; Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007; Ciucci et al., 2007, 2009; Scardochio, Trujillo-Pisanty, Conover, Shizgal, & Clarke, 2015).

These appetitive 50 kHz calls may be induced by a variety of positive behavioural contexts, including for example: anticipation and access to consumable food rewards (Burgdorf, Knutson, & Panksepp, 2000; Brenes & Schwarting, 2015), social scenarios involving play (Himmler, Kisko, Euston, Kolb, & Pellis, 2014), or exposure to male or female conspecifics (Sales, 1972; Knutson et al., 1998; Willey, Varlinskaya, & Spear, 2009; Willey & Spear, 2012). There is evidence that these various behavioural situations may differ in their capacity to induce both 50 kHz USVs call rate and may possess unique associations with emission of individual USV subtype proportions (Burke, Kisko,

Swiftwolfe, Pellis, & Euston, 2017; Mulvihill & Brudzysnki, 2018b). Regardless of the specific context, it is hypothesized that the degree of call induction (and in particular the amount of frequency modulation; FM) is reflective of a common underlying emotional state (Burgdorf, Panksepp, & Moskal, 2011; Barker, Simmons, & West, 2015; Simola & Brudzynski, 2018).

A similar behavioural measure of this emotional state often employed in the rat beside recording of USVs, is locomotor activity (Taracha et al., 2012, 2014; Garcia & Cain, 2016). Both of these behaviours are increased after application of drugs that directly stimulate the mesolimbic dopamine system. As such, USVs and motor activity feature prominently in many experimental paradigms centered around models of addiction and sensitization (Hooks, Jones, Smith, Neill, & Justice Jr., 1991; Hooks, Jones, Neill, & Justice Jr., 1992; Panksepp, Knutson, & Burgdorf, 2002; Browning et al., 2011; Maier, Abdalla, Ahrens, Schallert, & Duvauchelle, 2012; Mahler et al., 2013; Simola, Frau, Plumitallo, & Morelli, 2014; Barker et al., 2015). Several such studies have recently found that these two behavioural measurements respond differently to psychostimulant sensitization (Taracha et al., 2012, 2014; Ahrens et al., 2013; Costa, Morelli, & Simola, 2015; Garcia & Cain, 2016). In addition to a dissociation between calling and locomotor behaviours, this sensitization research has revealed extensive inter-individual variability in their expression (Taracha et al., 2012, 2014, 2016). In several studies that employed psychostimulants to investigate 50 kHz calling, the initial individual variability was used to screen out or categorize subjects (Wright, Gourdon, & Clarke, 2010, Wright, Dobosiewicz, & Clarke, 2012; Taracha et al., 2012, 2014, 2015, 2016; Ahrens et al., 2013).

The psychostimulant D-amphetamine (AMPH) has been used extensively to investigate expression and sensitization of 50 kHz USVs (Ahrens, Ma, Maier, Duvauchelle, & Schallert, 2009; Simola & Morelli, 2015; Taracha et al., 2016; Simola & Costa, 2018). Most knowledge about the possible brain areas involved in AMPH-induced 50 kHz calling has been inferred from targeted pharmacological studies (i.e., AMPH microinjections into the shell of the nucleus accumbens, NAcSh; Burgdorf, Knutson, Panksepp, & Ikemoto, 2001; Thompson, Leonard, & Brudzynski, 2006). The degree of involvement among various forebrain regions with this AMPH-induced USV sensitization has begun to be investigated using changes in the expression of transcription factors associated with immediate early genes (IEGs). The detection of well characterized IEG-associated inducible transcription factors such as Fos or Zif-268 (Zif) has been reliably used to index levels of neural activation associated with psychostimulant administration (Beckmann & Wilce, 1997; Steiner, 2010). The research into psychostimulant-induced brain activation has extended the findings of dissociation in behavioural sensitization between locomotor and 50 kHz calling activities (Costa et al., 2015; Hamed et al., 2016; Kaniuga et al., 2016).

These studies have revealed a complex network of associated brain systems possibly involved in the expression and sensitization of 50 kHz USVs. Importantly, a role of both cortical and subcortical forebrain structures in promoting the AMPH-induced 50 kHz USVs has been highlighted (Costa et al., 2015; Hamed et al., 2016; Kaniuga et al., 2016). In line with this, Costa and colleagues (2015) established a critical role of glutamatergic signaling for the acute and conditioned effects of AMPH-induced 50 kHz calling and AMPH-induced Zif expression. However, this research has either used

extensive sensitization protocols (> 2 AMPH injections) or overlooked possible inter-individual variation.

The purpose of the present study was to complement this earlier work by investigating the relationships between brain regions of interest and 50 kHz calling in minimally sensitized rats (two injections). Using this approach, it was hoped that the possible forebrain networks associated with the individual variability observed in 50 kHz calling after AMPH could be explored. We also sought to determine whether the dissociation of AMPH-induced behavioural sensitization observed between 50 kHz USVs and locomotor activity could be extended to a more general measure of ergometric activity. The current research ultimately aimed at determining if patterns of AMPH-induced gene regulation would correlate with 50 kHz USV behaviour across cortical and striatal forebrain regions. These regions included the medial prefrontal cortex (prelimbic and infralimbic), ventral striatum (nucleus accumbens core and shell), medial portion of the dorsal striatum, premotor cortex, and basolateral amygdala. We hypothesized that 50 kHz calling and general ergometric activity would be dissociable behavioural measures although both would show sensitization to two-injections of AMPH.

Methods

Subjects

A total of 31 male Long Evans rats (Charles River Laboratories, Saint-Constant, QC, Canada) were used in this study. Rats were 50 days old at their entry into the experiment with an average weight of 231g at the start and an average weight of 321g at the end of the experiment. In accordance with Brock University protocols for laboratory

handling, all rats were housed in pairs in polycarbonate cages (48 x 27 x 20 cm) with a plastic tube inside for hiding and an aspen block of wood for environmental enrichment. Cages were lined with dust-free corn cob bedding (Fisco Enterprises, Bolton, ON). Rats were given 5 days of acclimation time upon arrival into the animal facility before being entered into the study. All subjects maintained same cage-partner throughout the entire duration of the study. The housing room had controlled room temperature (21° C ± 2° C) and humidity (40-60%). Rats were housed with a maintained 12:12 h light/dark cycle and *ad libitum* access to food pellets (Ren's Feed & Supplies Limited, Oakville, ON) and filtered water. Behavioural procedures took place during the light phase of the cycle. All research protocols were approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

Equipment and materials

A Small Animal Movement Monitor (Colbourn Instruments, Lehigh Valley, PA) was used to record general ergometric activity of the rats. This system functions via an accelerometric sensor mounted under the movable stage the recording chamber sits upon. The output consists of force-displacement / time integral counts and is proportional to the ergometric activity of the rat. The sensitivity was arbitrarily set for 5 g × s per count. The rat's activity was recorded for both small and large activity discriminations. Small movement was detectable by a very low threshold for movement of the ergometric stage, while large movement was detected with a higher threshold for whole body movement on the stage. The counts for small and large movement were recorded as cumulated counts

within 10 min bins, and in addition to the recordings, the rats' behavior was simultaneously observed.

For all recordings of USVs the subjects were placed into a recording chamber (25 cm wide × 18 cm deep × 18 cm height polycarbonate cage) where USV production was recorded for 10 minutes. The recording chamber was filled with fresh corn cob bedding and was not re-used across rats, every rat received a new chamber each recording. The recording chamber sat atop the ergometric activity recorder allowing for emitted USVs to be recorded during the same time window with ergometric activity. USVs were recorded using an UltraSoundGate CM16/CMPA (Avisoft Bioacoustics, Glienicke, Germany) condenser microphone (working frequency range 2-250 kHz) located on top of the recording apparatus on a metal grate (approximately 25 cm from the animal). The microphone was connected via an UltraSoundGate 416 USB audio device (Avisoft Bioacoustics) to a computer (Dell PC) and recordings were made using multi-channel triggering hard-disk software (Avisoft RECORDER version 4.40). Acoustic data were recorded at a sampling rate of 250 kHz in 16-bit format. Analysis of USVs was done off-line using Avisoft SASLab Pro (version 4.40) and Sonotrack™ (Metris BV, Hoofddorp, The Netherlands) software (version 4.40).

A Nikon Eclipse 80i microscope equipped with a digital camera (Nikon DXM1200F) and Nikon ACT-1 software was used to visualize and capture images of the immunostained sections.

Procedure

All rats received habituation exposure to the general recording procedure (4 sessions), including the handling involved, the length of time in the recording chamber

and the setup of the room. All habituation sessions had USV production recorded along with small and large ergometric activity for 24 rats. After habituation, all subjects underwent the injection recording procedure (for experimental scheme see Figure 5-1). Briefly, subjects were placed into the recording cage and a 10 min baseline was recorded. After this 10 min period had elapsed the rat was removed from the recording cage and injected with *D*-amphetamine sulphate (dextroamphetamine, Sigma-Aldrich, Great Britain) at a dose of 2.5 mg/kg (s.c.) and then placed back into the recording chamber where any USV production was recorded for 40 minutes. Drug was dissolved and administered in 0.2 ml vehicle of sterile physiological saline. Ergometric activity was also recorded for both the 10 min baseline and 10 min post-injection time periods. All subjects underwent the two-injection protocol of sensitization (TIPS) (Valjent et al., 2010), whereby 6 days after the first injection of AMPH the rats receive a second equal dose. This protocol has been previously employed to sensitize both locomotor activity and 50 kHz USVs to AMPH in rats (Taracha et al., 2012). Injectable solutions were prepared fresh the day of injections.

The second injection recording used the exact same procedure as the first except after the injection recording itself the rats were placed into a holding cage for 50 minutes before the extraction of brain tissue took place so that tissue could be immunostained for Zif expression (for experimental scheme see Figure 5-1).

Zif immunohistochemistry

For extraction, subjects were deeply anaesthetized by an overdose of sodium pentobarbital (150 mg/kg) and transcardially perfused with 0.9% saline followed by chilled 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffered saline (PBS; pH 7.4).

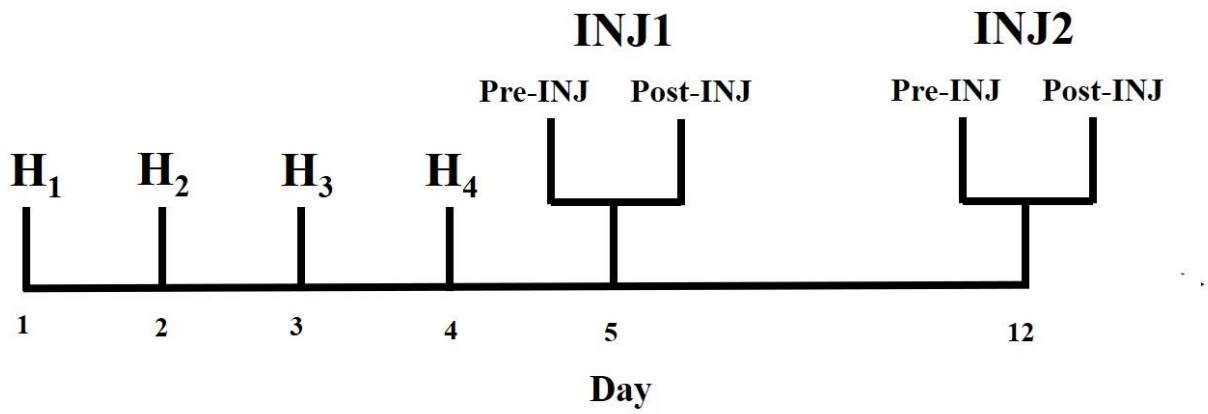

Figure 5-1. Scheme of experimental design. All timepoints used for behavioural measurements are illustrated. H_1 to H_4 represent days of habituation protocol, pre-INJ and post-INJ represent pre- and post- 10 min periods associated with each injection of AMPH across the two days of the TIPS protocol (INJ1 – first injection and INJ2 – second injection). For elaboration of TIPS protocol, see Methods section. At each timepoint ergometric movement activity (small and large movements) as well as 50 kHz USV emission were recorded.

Once removed from the skull brains were post-fixed in a 30% sucrose 4% paraformaldehyde solution at 4° C until equilibrated and then sectioned into coronal slices (40 μm thick) and stored in cryoprotectant at -20° C until the time of assay.

Free-floating brain sections were first washed in 0.1 M PBS, then in PBS-X (0.1 M PBS with 3% Triton X-100) before being bathed in a solution of 0.3% H_20_2 in 0.1 M PBS-X for 30 min at room temperature. Sections were then washed in PBS-X, blocked in 10% normal goat serum (Sigma-Aldrich) at room temperature for 1 h, and incubated at 4° C overnight in primary antibody (1:5000; zif268/EGR1 rabbit mAB; 15F7; Cell Signaling Technology, Inc., Danvers, MA) in PBS-X. The following day, sections were washed again in PBS-X and then incubated for 1 h in secondary antibody (biotinylated goat anti-rabbit IgG; 1:200; Vector Laboratories, Inc.) at room temperature. Sections were then washed thoroughly in PBS-X and were incubated in an avidin-biotin horseradish peroxidase complex (Vector Laboratories, Inc., Burlington, ON) for 1.5 h at room temperature. Horseradish peroxidase was visualized with the chromogen 3,3' Diaminobenzidine in a 3 M sodium acetate buffer containing 0.05% H_20_2 (Vector Laboratories, Inc.). Nickel was added to the chromogen solution to increase detection efficiency of stained cells. After a final series of washes in PBS-X, sections were mounted on slides and lightly counterstained with neutral red to facilitate identification of subregions. Mounted sections were then dehydrated in increasing ethanol concentrations (70%, 95%, 100%), placed in xylenes, and coverslipped using Permount mounting medium (Fisher Scientific, Inc., Hampton, NH). No immunoreactive cells were observed in control sections included in the assay that were not treated with the primary antibody.

Cell counting

Immunoreactive cell counts were obtained from the immunostained sections at 200x magnification in a 500 μm^2 area in each hemisphere of the prelimbic cortex (PL), infralimbic cortex (IL), nucleus accumbens shell (NAcSh), nucleus accumbens core (AcbC), dorsomedial striatum (DMS), primary motor cortex (M1), and basolateral amygdala (BLA). These areas were chosen for examination based on their established roles in Fos- and Zif-positive associations with AMPH generally (Rotllant, Márquez, Nadal, & Armario, 2010), and with AMPH-induced 50 kHz calling specifically (Costa et al., 2015; Kaniuga et al., 2016). Regions were identified in accordance with a stereotaxic atlas of coronal sections for the rat brain (Paxinos & Watson, 2007). PL sections used for counting were between 13.68 mm and 11.52 mm from interaural coronal plane; IL sections were between 12.72 mm and 2.52 mm from interaural plane; NAcSh sections were between 12.00 mm and 9.72 mm from interaural plane; AcbC sections were between 11.76 mm and 9.72 mm from interaural plane; DMS sections were between 11.76 mm and 7.68 mm from interaural plane; M1 sections were between 13.20 mm and 9.36 mm from interaural plane; BLA sections were between 7.44 mm and 5.52 mm from interaural plane. The average number of Zif-positive cells per hemisphere per brain region was used for analysis. The expression of Zif was used in the current study as it is constitutively expressed in the regions of interest with relatively high baseline levels, which make it most suitable for correlative analyses (Schlingensiepen, Lüno, & Brysch, 1991; Worley et al., 1991; Beckmann & Wilce, 1997).

Ultrasonic vocalizations analysis

USV calls were analyzed and the identification and characterization of USVs was accomplished as described previously in several papers (Brudzynski, 2009, 2015;

Mulvihill & Brudzynski, 2018a, 2018b). Briefly, 50 kHz USVs had peak frequencies between 35 and 90 kHz, were typically less than 100 ms in duration, and had varying degrees of frequency modulation. 22 kHz USVs (20 - 30 kHz) were rare or absent. All spectrograms were initially screened for occurrence of 22 kHz USVs. Given the virtual absence of 22 kHz USVs this call type was omitted from the analysis. The two analysis programs were used for distinct and non-overlapping analyses. Reliability analysis for determining number of 50 kHz calls between the two programs indicated that performance of the Sonotrack progam is comparable to a competent experimenter performing manual detection using Avisoft program (Intraclass correlation coefficient = .978, 95% CI: .952, .990). Sonotrack-generated spectrograms were utilized for preprogrammed automatic screening which determined number of USVs across entire recordings as well as mean sound frequency of individual calls. The call rate and mean sound frequency of calls determined from Sonotrack-generated spectrograms were used for all analyses investigating USV calling across time points. Avisoft SASlab-generated spectrograms were manually screened for 50 kHz USVs for the first 2 min after the second AMPH injection. These SASlab spectograms were used for determining call subtypes and duration of calling, and only within this time period across subjects. For any automatic detection of 50 kHz calls by Sonotrack, a bandpass filter was employed to reduce background noise (low and high cut-off frequencies of 35 and 90 kHz respectively. All spectrograms were generated using a fast Fourier transform (512 FFT-length, 100% frame, Flat Top window, and 75% time window overlap), at 488 Hz of frequency resolution.

USV subtype determination was based on sonographic shape in a similar manner as described previously (see Mulvihill & Brudzynski, 2018b). 50 kHz calls were classified into the flat subtype if they appeared to have a relatively constant frequency (bandwidth < 6 kHz). If the calls were frequency-modulated they were classified as either trill or non-trill subtypes. For calculating FM to flat ratios all subtypes with frequency modulation (both trill and non-trill FM calls) were counted and divided by the number of flat 50 kHz USVs. Manual screening of 50 kHz USVs was accomplished by one trained experimenter.

Statistics

All statistical analyses were performed using SPSS Statistics (version 20, IBM Corporation). Where suitable, repeated-measures analyses of variance (ANOVAs) were used to assess possible differences in each behavioural measure (small and large movements, and 50 kHz USVs) across each time point (pre- or post-AMPH injection across either INJ1 or INJ2, see Figure 5-1). Each INJ (INJ1 and INJ2) had within it a pre-injection and post-injection recording period. The equal time periods 10 min pre-injection and 10 min post-injection were used to look at the acute effect of AMPH (PrePost). Differences between injections (INJs) were used to assess forms of behavioural sensitization. Due to incomplete ergometric activity or cell-staining data pairwise exclusions were used for analyses involving ergometric activity or Zif-staining data. Given that all analyses were entirely within-subjects this resulted in different rats being excluded for different analyses. For analyses involving ergometric activity 6 subjects were excluded (remaining n = 24). For analyses involving Zif-stained cell counts 6 subjects were excluded (remaining n = 24). For measuring bivariate parametric

associations Pearson's product-moment correlation coefficient (*r*) was used. Wherever possible and necessary non-parametric distributions were corrected using a logarithmic transformation using the natural base of *e* after a linear transformation of +1. For variables where this correction was necessary but not possible (i.e., Zif-immunostained cell counts) bivariate associations were measured using Spearman's rank correlation coefficient (*rs*).

For purely exploratory purposes a categorization was employed to specifically investigate a subgroup of the sample defined by duration of calling after the second AMPH injection. This categorization was based on whether the rat spent longer than 2.5% of the sampled time emitting 50 kHz USVs following the second AMPH injection (10 standard deviations from the average duration of calling at baseline sampling). Due to the extreme positive skew of the variable, performing this categorization effectively captured the majority of the variance observed in the group of animals that had this duration of calling. This subgroup of high duration callers (n = 11) was used only for exploratory analyses investigating non-parametric associations of immunostained brain regions.

For any given family of analyses, the type I error rate was stringently controlled for using a Bonferroni correction. For the non-parametric correlation analyses that involved 21 correlations, this resulted in a corrected *p* value of .0024 (Bonferroni $p = .05/21$). Thus, only *rs* with a *p*-value below this corrected value are reported as significant in the results. Where suitable, bias-corrected and accelerated bootstrap (BCa) confidence intervals are reported utilizing 1000 bootstrap samples.

Results

Amphetamine administration and the sensitization of measured ergometric activity

As anticipated, a (2 x 2) repeated measures ANOVA on large movements across the whole sample (n = 24) found a significant main effect of drug injection (INJ1 vs INJ2; $F_{1,23} = 28.94$, $p < .001$), significant main effect of timepoint (pre-injection versus post-injection, Pre-Post; $F_{1,23} = 164.77$, $p < .001$), and a significant interaction effect (INJ x PrePost; $F_{1, 23} = 22.13$, $p < .001$). Observed large movements (large scores of ergometric activity) were greater in INJ2 recordings than in INJ1, and in both INJs was found to be greater post-injection than pre-injection. A simple effects analysis found that the large movements were greater for INJ2 than INJ1 at both pre-injection ($t_{23} = 3.10$, $p = .005$) and post-injection timepoints ($t_{23} = 5.30$, $p < .001$). This provides evidence the TIPS protocol using amphetamine significantly sensitized large movements (see Figure 5-2).

A similar (2 x 2) repeated measures ANOVA on small movements using the same time points (n = 24) found no significant main effect of INJ ($F_{1,23} = .309$, $p = .584$) but there was a significant main effect of PrePost ($F_{1,23} = 14.68$, $p = .001$). There was additionally a significant interaction effect found for INJ x PrePost ($F_{1,23} = 8.37$, $p = .008$). Small movement activity recorded post-injection was found in significantly higher levels compared to pre-injection levels only in INJ1, likely as a result of the pre-injection small movement activity recorded in INJ2 being sensitized by the TIPS protocol (see Figure 5-3). A simple effects analysis found pre-injection recorded small movements were significantly greater in INJ2 compared with INJ1 ($t_{23} = 2.38$, $p = .026$), with no difference for post-injection timepoint ($t_{23} = 1.88$, $p = .072$).

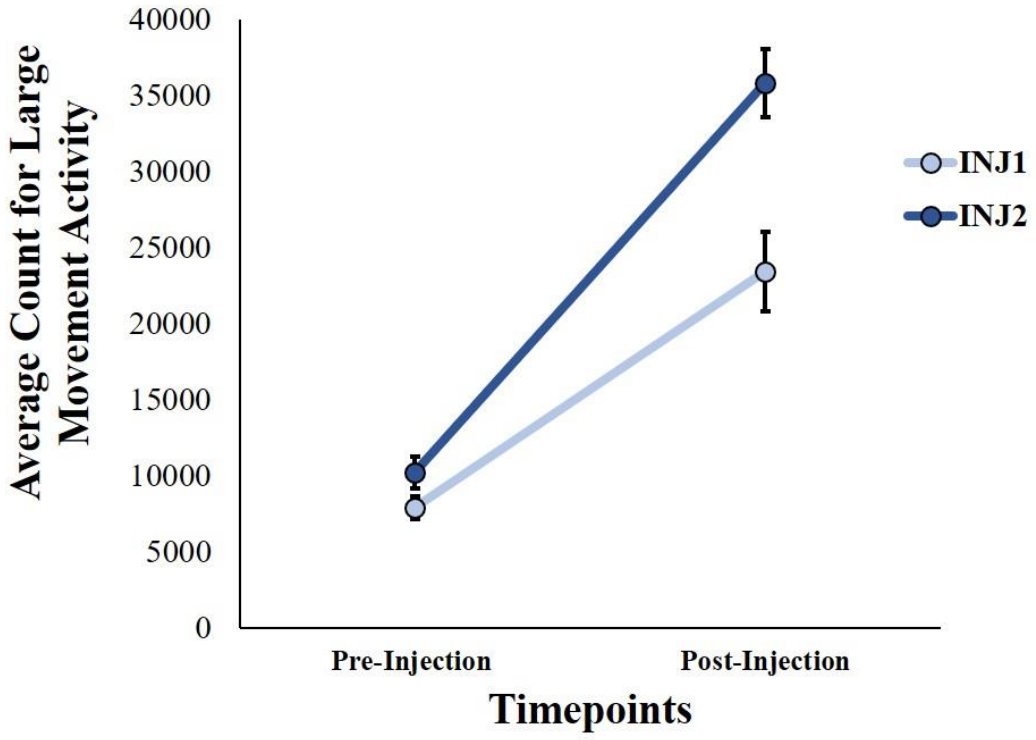

Figure 5-2. Comparison of average counts for large movement activity across recorded timepoints for both AMPH injections (INJ1 and INJ2). INJ2 was found to induce greater average large movement activity acutely after AMPH (post-injection average across INJs was greater than pre-injection average). INJ2 was found to have greater average large movement activity across both pre-injection and post-injection timepoints relative to INJ1. Both main effects (pre-injection versus post-injection and INJ1 vs INJ2) were significant at $p < .001$. There was also a significant interaction effect found with the acute effect of AMPH on large movement activity being greater for INJ2 than INJ1 at $p < .001$ level. Results are represented as means ± SEM.

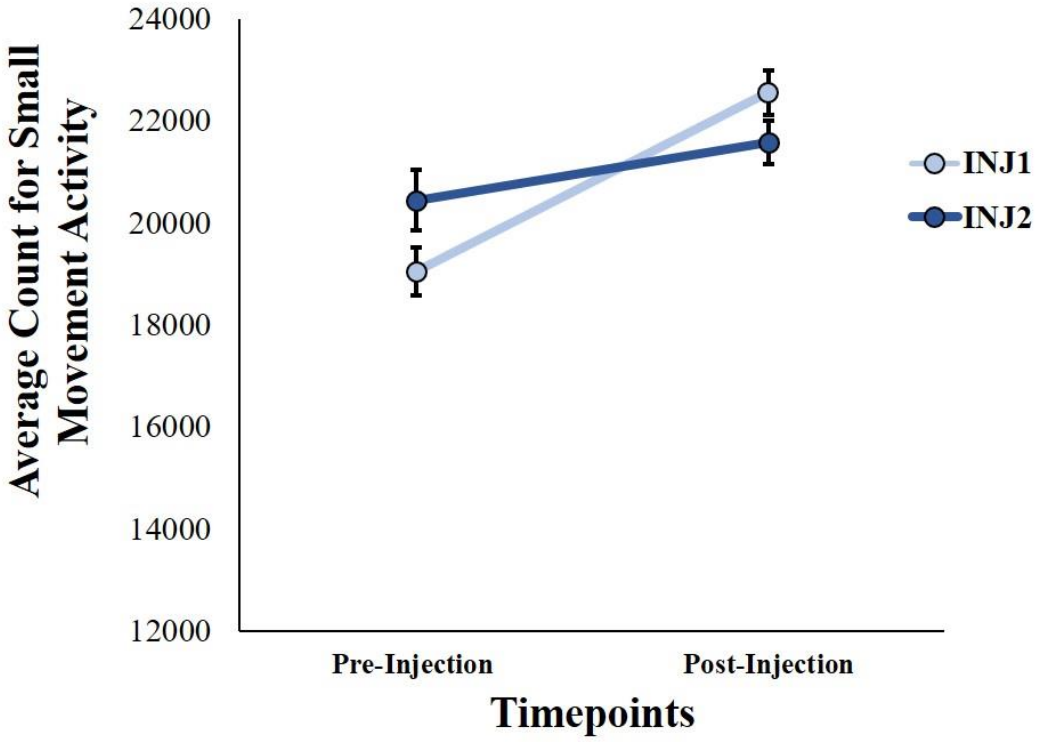

Figure 5-3. Comparison of average counts for small movement activity across recorded timepoints for both AMPH injections (INJ1 and INJ2). Acute effect of AMPH was found to induce greater average small movement activity when compared with pre-injection time period after AMPH for both INJ1 and INJ2 (post-injection average across INJs was greater than pre-injection average). This main effect of PrePost was significant at $p < .01$. There was no main effect found for INJs for small movement activity. There was a significant interaction found, with INJ2 differing from INJ1 for pre-injection timepoint only. This interaction effect was significant at $p < .01$. Results are represented as means ± SEM.

Neither small nor large movement activity recorded post-injection at INJ2 significantly correlated to time spent calling at the same time point (small: r = .005, p = .985; large: r = .322, p = .179). Investigating the association of the sensitized movement activity measures (determined by calculating the difference between INJ2 and INJ1 for both small and large movements in post-injection period) revealed a significant negative relationship (r = -.419, p = .042). Individuals with a higher difference between injections for either small or large movement activity measures exhibited a smaller difference for the other corresponding measure. This disassociation between sensitized movement measures partially mapped onto time spent calling after INJ2, with small movement sensitization possessing a significant negative relationship with time spent calling (r = -.447, p = .048). No significant relationship was found with the large movement sensitization measure (r = .259, p = .270).

Amphetamine administration and the sensitization of 50 kHz USV calling

A (2 INJ x 2 PrePost) repeated measures ANOVA on 50 kHz USV call rate (number of USVs/min) across the whole sample (n = 24) found significant main effects of INJ ($F_{1,23}$ = 113.62, p < .001) and PrePost ($F_{1,23}$ = 194.18, p < .001), as well as a significant INJ x PrePost interaction ($F_{1,23}$ = 122.18, p < .001). A simple effects analysis found that only the 10 min after the AMPH injection (t_{23} = 10.92, p < .001), not the 10 min prior to injection (t_{23} = 1.11, p = .279), significantly differed between injections (see Figure 5-4). The second AMPH injection induced significantly more 50 kHz USVs per min (M = 89.2, SD = 30.7) than the first AMPH injection (M = 31.6, SD = 17.3). No bivariate relationships were found between the sensitization measure of 50 kHz USV rate

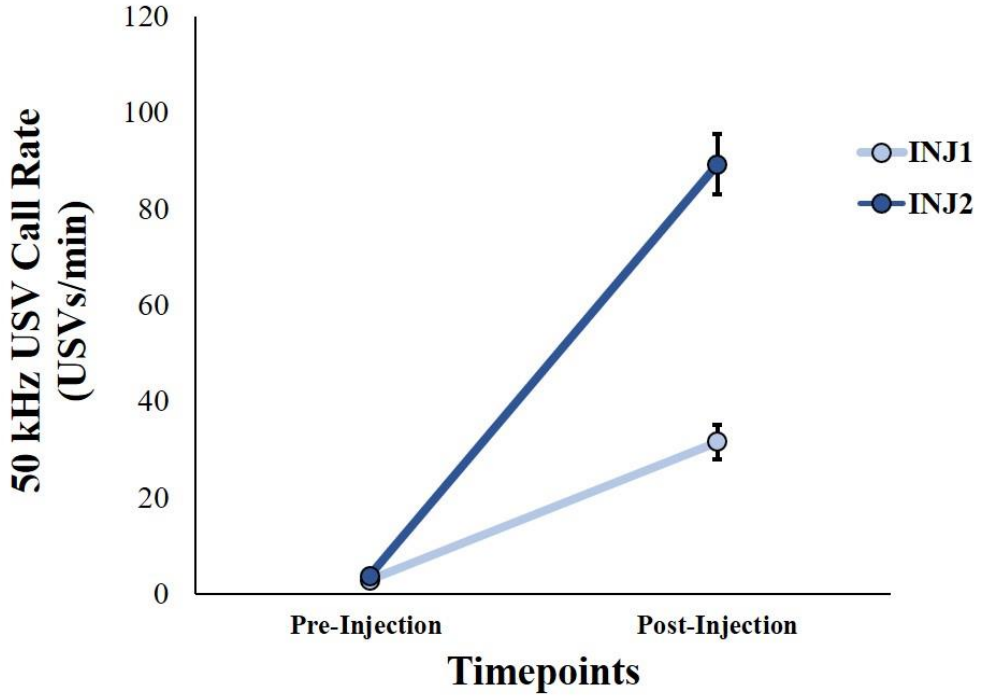

Figure 5-4. Comparison of average counts for 50 kHz USV call rate (USVs/min) observed across recorded timepoints for both AMPH injections (INJ1 and INJ2). The injection of AMPH induced significant 50 kHz USV emission compared to pre-injection baseline in both INJ1 and INJ2. However, AMPH at INJ2 induced a greater degree of 50 kHz USV calling when compared with AMPH at INJ1. Both main effects (pre-injection versus post-injection and INJ1 vs INJ2) were significant at $p < .001$. There was also a significant interaction effect found with the acute effect of AMPH on 50 kHz call rate found to be greater for INJ2 than INJ1 at $p < .001$ level. Results are represented as means ± SEM.

(difference between post-injection call rate between INJ1 and INJ2) and sensitization of either small (r = -.115, p = .594) or large (r = .281, p = .184) movement activity counts.

Calling prior to the first AMPH injection (pre-injection INJ1) was found to be significantly positively correlated with time spent calling after AMPH injection at INJ2 (r = .531, p = .023). This indicates a significant relation between a baseline predisposition to emit 50 kHz USVs and the time spent emitting after the TIPS protocol. Furthermore, there was no significant correlation found for the measure of sensitization of 50 kHz calling and time spent calling post-injection at INJ2 (r = .106, p = .598). This may suggest that the measure of time spent calling immediately after the injection at INJ2 reflects more of a dispositional trait towards emitting calls than a product of the sensitization protocol.

The acoustic parameter of average sound frequency for individual calls was assessed across the same time points as 50 kHz call rate using a (2 INJ x 2 PrePost) repeated measures ANOVA. The administration of AMPH was found to have significantly increased average sound frequency of emitted calls (main effect of PrePost: $F_{1,23}$ = 95.46, p < .001). There was additionally a significant main effect of INJ ($F_{1,23}$ = 15.01, p = .001). A significant INJ X PrePost interaction effect was also found ($F_{1,23}$ = 4.43, p = .047), which indicates that the difference between INJs was not equal between the pre- and post-injection periods (see Figure 5-5). A simple effects analysis indicated that average sound frequency was significantly higher in INJ2 compared with INJ1 only for the post-injection (t_{23} = 3.96, p < .001) but not the pre-injection ((t_{23} = 1.30, p = .205) timepoint. Post-injection INJ2 was found to have a higher average sound frequency (M = 64.39 kHz, SD = 5.7 kHz) than the post injection recording at INJ1 (M = 59.34 kHz, SD =

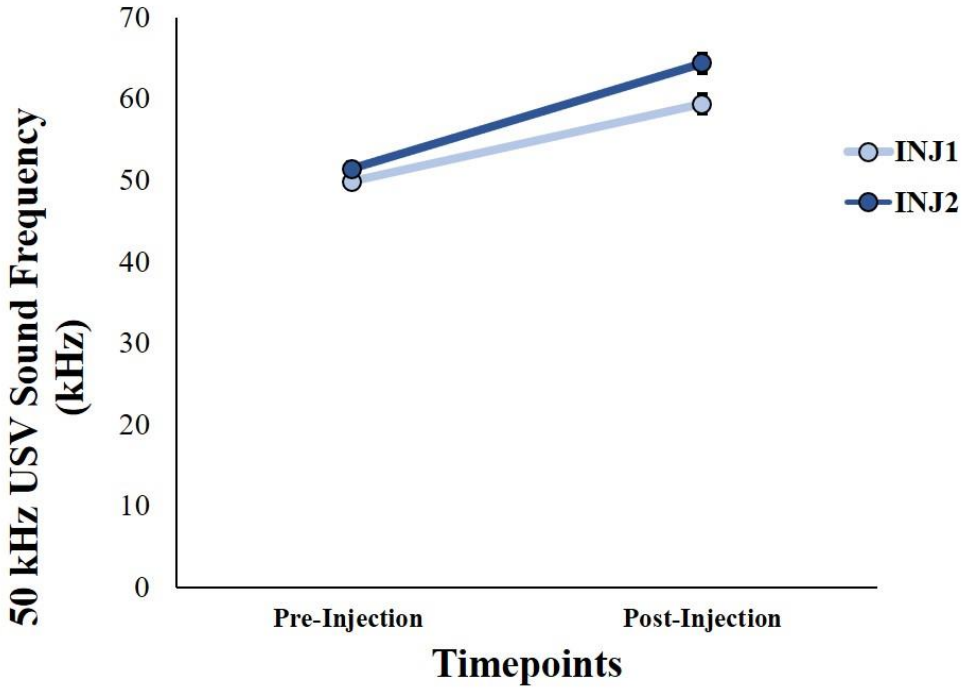

Figure 5-5. Comparison of the average sound frequency of individual 50 kHz calls observed across recorded time points for both AMPH injections (INJ1 and INJ2). Injection of AMPH significantly increased average sound frequency of individual 50 kHz calls recorded in both INJs. The second AMPH injection (INJ2) increased average sound frequency to a greater degree than INJ1. Both main effects (pre-injection versus post-injection and INJ1 vs INJ2) were significant at $p < .001$. The interaction effect was also significant with acute effect of AMPH producing greater increase on sound frequency of calls for INJ2 than INJ1 at $p < .05$ level. Results are represented as means ± SEM.

5.7 kHz). An analysis of the relationship between this acoustic parameter and time spent calling after AMPH injection at INJ2 indicated that, during the same recording window, there is no significant relationship between average sound frequency of individual calls and time spent emitting 50 kHz USVs (r = .161, p = .422). Additionally, there was no significant relationship found between a measure of sound frequency parameter sensitization (INJ2-INJ1 post-injection difference) and time spent calling after AMPH in INJ2 (r = -.344, p = .079).

50 kHz USV subtype characterization of time spent calling at INJ2

An investigation of the relation of flat, trill, and non-trill FM 50 kHz USV subtype proportions with the variable of time spent calling was done via bivariate correlations. This analysis indicates that time spent calling was significantly positively correlated with the percent of trill calls, significantly negatively correlated with percent of flat calls, and had no relation to percent of non-trill FM calls (see Table 5-1). Thus, the character of 50 kHz USV emission associated with time spent calling is mostly FM and in particular calls of the trill variant.

Non-parametric correlation analysis of brain region expression of Zif and 50 kHz calling

The characterization of time spent calling after the TIPS procedure indicated that this variable reflected a greater relation to baseline predisposition to emit USVs than any of the measured metrics of sensitization. However, there were no significant first-order Spearman's correlations found between time spent calling and Zif expression in any of the investigated brain regions of interest. Investigating the Spearman's correlations among examined brain regions revealed possible patterns of Zif-positive brain expression

following AMPH. Significant positive relationships were found between the DMS and IL, and DMS and BLA regions (see Table 5-2).

Table 5-1. Correlation matrix of bivariate coefficients between percent of each 50 kHz USV subtype and time spent calling to characterize the type of calling predominantly reflected by this variable.

		1	2	3	4
1. Time spent calling	Pearson's r				
		—	-.614***	.518**	-.181
2. Percent of flat calls					
3. Percent of trills					
4. Percent of non-trill					
FM					

* p < .05, ** p < .01, *** p < .001.

In an exploratory analysis using only rats characterized by their duration of calling after AMPH in INJ2 (see methods for details), there was a positive correlation found between the PL cortex and NAcSh region in addition to the IL cortex and the DMS (see Table 5-3). There were a number of possible intercorrelations beyond these noted structures observed within both groups; however, the coefficients did not remain statistically significant after correction of familywise error. The Spearman's correlations for the group of rats with a low duration of calling is included in Table 5-4.

Table 5-2. Correlation matrix of Spearman's coefficients for Zif expression after AMPH at INJ2 between each of the measured brain regions of interest for all subjects (n = 24).

	1	2	3	4	5	6	7
1. PL		.526	.444	.394	.288	-.197	.078
2. IL			.394	.267	.671***	.143	.601
3. NAcSh				.469	.387	.105	.269
4. AcbC					.199	.237	.066
5. DMS						.246	.696***
6. M1							.371
7. BLA							

* p < .05, ** p < .01, *** p < .001.

Table 5-3. Correlation matrix of Spearman's coefficients for Zif expression after AMPH at INJ2 between each of the measured brain regions of interest within the high duration of 50 kHz calling subgroup (n = 11).

	1	2	3	4	5	6	7
1. PL		.427	.841***	.482	.454	-.032	.061
2. IL			.351	.281	.856***	.305	.671
3. NAcSh				.757	.304	.184	-.133
4. AcbC					.062	.476	-.029
5. DMS						.082	.707
6. M1							.305
7. BLA							

* p < .05, ** p < .01, *** p < .001.

Table 5-4. Correlation matrix of Spearman's coefficients for Zif expression following AMPH at INJ2 between each of the measured brain regions of interest within the low duration of 50 kHz calling group (n = 13).

	1	2	3	4	5	6	7
1. PL		.684	.210	.289	.149	-.340	.095
2. IL			.623	.269	.629	.035	.528
3. NAcSh				.378	.503	.072	.551
4. AcbC					.341	.166	.209
5. DMS						.423	.692
6. M1							.454
7. BLA							

* p < .05, ** p < .01, *** p = .001.

Discussion

In the present study we obtained measures of ergometric activity, 50 kHz calling, and expression of Zif protein across several cortical and subcortical brain regions after a TIPS protocol using AMPH. The results suggest that although both movement activity and 50 kHz USV measures did show aspects of sensitization after the TIPS protocol these two behavioural measures were distinct, dissociable and not positively correlated. Moreover, the present study found a measure of 50 kHz USV emission (specifically time spent calling after second AMPH injection) which did not correlate with any measure of AMPH sensitization. This 50 kHz USV measure was used to explore patterns of Zif expression across several brain regions using a non-parametric correlative analysis. This correlative analysis revealed possible patterns of Zif expression between IL and DMS and BLA and DMS brain regions. Within subjects characterized by duration of calling

(greater than 3 s in duration of 50 kHz USVs) there was an additional relation of Zif expression between PL and NAcSh regions observed. This study dissociates 50 kHz USV emission from general ergometric activity and highlights the possible association of frontal cortex and striatal brain activity with AMPH-induced 50 kHz USV emission.

Several studies utilizing injections of psychostimulants (including AMPH) to investigate 50 kHz USVs and locomotor activity have found that these behaviours represent distinct behavioural responses (Maier et al., 2012; Taracha et al., 2012, 2014; Garcia & Cain, 2016). The results of the current study support the hypothesis that this dissociation observed between locomotor activity and emission of 50 kHz USVs can be extended to a more general measure of ergometric activity. This adds support to the notion that 50 kHz USVs of the rat may be reflective of the operation of a distinct brain system that responds to repeated psychostimulant exposure in a manner separable from general movement based behaviours. As expected, there was evidence of behavioural sensitization for both movement activity and 50 kHz calling across the TIPS protocol. However, neither small nor large movement activity counts recorded after AMPH were found to correlate with 50 kHz USV call rate emitted after AMPH application. Moreover, no relationship was found between the sensitization of large or small movements and the sensitization of 50 kHz USV call rate. In addition to call rate, the average sound frequency of individual calls was also found to be sensitized across the TIPS protocol.

In the current study we utilized the measures of ergometric activity (including the movement measures of behavioural sensitization) and measures of 50 kHz USV behavioural sensitization (call rate and average sound frequency) to characterize the variable of time spent calling after the second AMPH injection. This time spent calling

was found to not significantly correlate with either small or large movement activity, although a negative relationship was found with sensitization of small movement activity. Time spent calling as a behavioural measure was also not related to average sound frequency or the sensitization of 50 kHz call rate. However, time spent calling was significantly correlated to baseline call rate (recorded prior to AMPH for INJ1). Thus, our data suggest that time spent calling may reflect a behavioural measure associated with 50 kHz USV emission partially dissociable from other metrics of psychostimulant sensitization. This behavioural measure of length of time associated with USV emission has been employed previously in investigations into 22 kHz calls (Brudzynski, Iku, & Harness, 2011).

Previous research has established that repeated injections of AMPH produce significant changes in gene regulation within striatal and cortical brain regions in a dose-dependent fashion when compared with vehicle controls (Nguyen, Kosofsky, Birnbaum, Cohen, & Hyman, 1992; Rotllant et al., 2010). The detection of well-established markers of neuronal activity, the Fos and Zif proteins, have been used to examine brain region activity associated with AMPH-induced 50 kHz calling in repeated administration context (Costa et al., 2015; Kaniuga et al., 2016). Moreover, beyond Fos and Zif expression, the IEG transcription factor Arc (associated with learning and memory) has been utilized in a model of neuroplastic changes associated with repeated AMPH treatment and sensitized 50 kHz calling (Hamed et al., 2016). However, few studies to date have investigated direct associations between the observed psychostimulant induced gene regulation and 50 kHz USVs using inter-individual differences. Kaniuga and colleagues (2016) found evidence of a relationship between Fos-staining and 50 kHz

calling only within rats classified as high-responders to the TIPS protocol and specifically for frequency modulated calls. Fos-positive cell counts within the nucleus accumbens and ventral tegmental area correlated with frequency modulated calls induced by AMPH. The present results with Zif expression did not find any relationship between nucleus accumbens cell staining and 50 kHz calling. These differences in findings may be a result of the current inclusion of total 50 kHz calling as opposed to only frequency modulated and the use of minimal sensitization (two injections of AMPH rather than several daily injections). It is also possible that these contrasting results reflect the differential role of Fos and Zif in AMPH induced gene induction (Nguyen et al., 1992; Wang, Smith, & McGinty, 1995).

The expression of Arc protein after a second injection of AMPH in rats was found to be correlated among the central amygdala, nucleus accumbens (both core and shell; Hamed et al., 2016). In the present study we did not assess the central amygdala but instead investigated the BLA which is known to receive dopaminergic modulation (Rosenkranz & Grace, 1999). There was a significant positive association found between the BLA and DMS in the whole sample. This may highlight the interconnected nature of the dorsal striatum with prefrontal and limbic circuitry. However, the lack of any prominent associations between BLA and ventral prefrontal regions observed in the current study was surprising. The prelimbic and infralimbic cortices were chosen as regions of interest in part because of their anatomical and functional relation with limbic associated brain structures including the BLA (Cardinal, Parkinson, Hall, & Everitt, 2002; Vertes, 2006; Roy et al., 2012). The glutamatergic input from medial prefrontal regions into the BLA, although subject to modulation by dopamine, is thought to be

inhibitory via the recruitment of inhibitory interneurons (Rosenkranz & Grace, 2002). In the present study there was no direct association found between BLA and investigated prefrontal regions (PL and IL).

The present study did find associations between the PL and IL with medial portions of the ventral and dorsal striatum respectively when analyzed in subjects characterized as high in duration of time spent emitting AMPH-induced USVs. These PL and IL cortices have been suggested to form a ventral component of the rat frontal cortex associated with integrating internal physiological states in the guidance of behaviour (Ongur & Price, 2000; Heidbreder & Groenewegen, 2003).

It must be noted that a major limitation of the methods utilized in the current study relate to the inability to determine the mechanism associated with the specific cortical and striatal relationships observed. It is not clear if there is any functional relation between the structures themselves or if the transcription factor induction by AMPH is similarly coincident. In aggregate, the results of the present study highlight the possible importance of corticostriatal regional activity in the expression of 50 kHz USVs after AMPH. The involvement of medial prefrontal areas with 50 kHz USV emission is generally consistent with the known involvement of the ascending mesocorticolimbic dopamine system (Burgdorf et al., 2007). While it has been established that these medial prefrontal cortices receive dopaminergic modulation, the exact relation of these regions in 50 kHz emission remains unknown (Seamans & Yang, 2004). More extensive investigations are needed to directly assess the role of prefrontal areas in AMPH-induced 50 kHz calling.

One important aspect for interpretation of the current study's results is the specific relation of the inducible transcription factor utilized (Zif) and the neurochemical alterations induced by AMPH. The systemic application of AMPH activates a multitude of monoaminergic systems throughout the nervous system including the catecholamines dopamine and noradrenaline and the indolamine serotonin (Sulzer, Sonders, Poulsen, & Galli, 2005; Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007). The induced expression of Zif appears to require co-activation of these different neurotransmitter systems (O'Donovan, Tourtellotte, Millbrandt, & Baraban, 1999). A central governing role in Zif expression has been proposed for the noradrenergic projections from the locus coeruleus (Bhat & Baraban, 1992; Cirelli, Ponpeiano, & Tononi, 1996). Additionally, Zif expression may predominantly reflect activation of D_1 receptors and thus may not adequately reflect all possible brain region activity relationships (Moratall, Robertson, & Graybiel, 1992; Wang & McGinty, 1995). This aspect underlines the utility in investigating a variety of inducible transcription factors associated with a given experimental paradigm. Intriguingly, using an extensive mapping of neurochemical effects associated with AMPH-induced 50 kHz USVs, Hamed and colleagues (2016) found evidence of an association between norepinephrine concentration in the nucleus accumbens and 50 kHz calling. There is evidence found in mice that norepinephrine within the prefrontal cortex is actually critical for AMPH-induced dopamine release within the nucleus accumbens (Ventura, Cabib, Alcaro, Orsini, & Puglisi-Allegra, 2003). These findings may tempt the speculation that given the importance of norepinephrine for induction of Zif expression (Bhat & Baraban, 1992), the expression observed in the

present study may reflect the same underlying neurochemical effects observed by Hamed and colleagues (2016).

There are several limitations specific to the current study that require comment. Due to our interest in the direct association between brain Zif expression and the observable behaviour of 50 kHz calling we utilized a within-subjects focused sample. This sample was exposed to one dose-procedure with no vehicle control. Vehicle controls were conducted in relatively small numbers simply to determine efficacy of AMPH dose and for determining particular aspects of the immunostaining assay. There was no vehicle control sample equivalent to the experimental sample in size included in the primary assay of interest to conserve sample size. Additionally, the use of *ex vivo* methods (immunostaining for transcription factors) allows for very indirect measurement of brain state putatively associated with the behaviour of interest. Future research may utilize more systematic *in vivo* techniques to uncover active regional associations more closely associated with 50 kHz calling.

Conclusions

The individual variability among rats in the expression of 50 kHz USVs is observed even after minimal sensitization. In the present study we demonstrated that the effect of a two-injection sensitization with AMPH differentially affects measures of ergometric activity and 50 kHz USV emission. Moreover, we found that after the second AMPH injection there was considerable inter-individual variability in time spent calling which was dissociable from other measures of sensitization. Within the whole sample following the second AMPH injection, there was correlative evidence of induced Zif-immunostaining associations between the IL and DMS, and DMS and BLA brain regions.

Given that time spent calling after the second AMPH injection was significantly predicted by a subject's baseline predisposition to emit 50 kHz USVs it was used to characterize a group of rats for exploratory analysis. When characterized according to duration of calling after the second AMPH there were patterns of Zif expression observed among prefrontal and striatal brain regions in rats with a longer duration of calling. Within these rats there were significant relationships in levels of Zif expression between PL and NAcSh and IL and DMS observed. The exact mechanism and extent of associations between these brain regions, their role in 50 kHz USV emission, and the possible involvement of other brain regions not investigated in the current study requires future research.

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Chapter 6: General Discussion and Conclusion

My research findings suggest that the 50 kHz USVs of the adult male Long Evans rat may reflect activity of a discrete subcomponent of the ascending mesocorticolimbic dopamine system. This subcomponent for emotional arousal in the brain may be activated by both pharmacological and behaviourally relevant means. This emotional arousal may be expressed behaviourally in a variety of forms; however, 50 kHz USV emission offers a particularly rich measure. My research characterized 50 kHz calling in several contexts: spontaneous calling, calling following application of AMPH, calling associated with social and non-social behavioural contexts, and calling associated with dopamine signaling within the nucleus accumbens.

My findings from chapter 2 indicate that the 50 kHz USVs observed following systemic AMPH application are significantly predicted by an individual rat's baseline predisposition to emit. Moreover, this predictive utility of calling trait is distinct from other behavioural measures associated with dopamine (liking and wanting). This chapter provided support to the notion that the individual differences in USV response to AMPH reflect both aspects of an individual's approach motivation and predisposition to vocalize. Moreover, these relations of individual behavioural predictors and response to AMPH were not in the context of chronic administration or sensitization. Baseline emission of 50 kHz USVs was found to provide additional information beyond a general measure of motivation and this indicates functional heterogeneity in the brain systems activated by acute systemic AMPH.

My findings from chapter 3 indicated that social and non-social contexts differentially induce 50 kHz USV subtypes. Social contexts increase the degree of

frequency modulation observed among individual calls while non-social contexts have a greater proportion of constant frequency calls. Importantly, the context most capable of inducing 50 kHz call emission also had the greatest selective increase on frequency modulated call types. Moreover, across all behavioural contexts antagonism of dopamine receptors successfully blocked stimulus-induced USV emission. This suggests that although 50 kHz call subtypes may be modified by behavioural context in their emission characteristics they all ultimately may reflect the function of a single underlying brain system.

My findings from chapter 4 demonstrated that local dopamine injections into the NAc brain structure were sufficient to induce 50 kHz USV expression compared with vehicle. Additionally, although there were notable differences in their efficacy to induce frequency modulation between dopamine and AMPH these differences were minimized by antagonism of the dopamine reuptake transporter. Of note was the finding that using only intracerebral application of dopamine there was a positive linear relationship between calling and frequency modulation. This suggests that even though localized in a terminal area of the ascending dopamine system the increase of dopamine was sufficient to induce 50 kHz calling with frequency modulation. This suggests that the frequency-modulation increase observed by AMPH application is at least partially aligned with the actions of the native transmitter.

My findings from chapter 5 indicate that the 50 kHz calling emitted after systemic AMPH application can be disassociated from a general measure of ergometric movement and measures of sensitization (both for movement and 50 kHz calling). Investigations of Zif expression across several brain regions of interest failed to find any direct relation

with behavioural responses to AMPH. However, in an exploratory analysis it was found that within a subgroup of rats characterized by extreme duration of 50 kHz calling after AMPH there were significant relationships found among prefrontal and striatal brain regions in their Zif expression. The possible involvement of prefrontal circuitry in the behavioural expression of emotional arousal is consistent with the notion of this system functioning to alter the total state of the organism.

Understanding the role of reward-related behavioural phenotypes in acute AMPH-induced 50 kHz USVs

Much of the interest in behavioural phenotypes and the response to psychostimulants results from models of drug addiction, gambling, and other pathological states of approach motivation. As an unconditioned behavioural response to psychostimulants the emission of 50 kHz USVs in rats has proven to be very useful in supplementing such models (Barker, Simmons, & West, 2015).

Many longstanding conceptions of approach motivation hold that it is oriented towards external stimuli (goal objects) and requires an associated positive affective state (Lang & Bradley, 2008). This formulation was largely adopted as part of the broader biphasic approach to animal motivation which posited all motivated behaviours could be viewed as approach or withdrawal behaviours (Schneirla, 1959). This dichotomous approach to motivation may be extended to the organization of emotional systems, whereby emotion results from activity in either appetitive or defensive motivational systems (Bradley, 2000; Lang & Bradley, 2008). This appetitive motivational system in most conceptions is largely dependent on the functioning of the mesolimbic dopamine system (Berridge & Robinson, 1998; Robinson & Berridge, 2000; Alcaro et al., 2007;

Hoebel, Avena, & Rada, 2008). It should be mentioned that similar frameworks, such as Gray's (1981) behavioural inhibition/activation systems, have been employed extensively in investigations of human personality and temperament (Carver & White, 1994; Trofimova & Robbins, 2016).

Alternatively, approach motivation has been conceptualized to not necessarily require an evoking stimulus or affective state and instead is proposed to arise from internal processes at the trait or state levels (Panksepp, 2005; Harmon-Jones, Harmon-Jones, & Price, 2013). In this sense it is defined as the 'impulse to go towards' without any specified valence while still being generally associated with the ascending mesolimbic dopamine system (Panksepp, 2005, 2011). Thus, regardless of the specific framework adopted the manifestation of approach motivated behaviour reflects to some degree the activity of the ascending mesolimbic dopamine system. My results from chapter 2 found that individual differences in approach motivation do significantly predict the USV response to acute AMPH. Moreover, individual differences in baseline USV emission also significantly predicted this AMPH-induced USV response. The finding that these two behavioural predictors largely did not overlap in their relation to the 50 kHz USVs emitted following AMPH indicates a meaningful dissociation between USVs and approach motivation. These findings suggest functional utility in the two behavioural measures in experimental paradigms involving AMPH. These findings may additionally, and more speculatively, offer evidence for conceptual delineation between functions of the mesolimbic dopamine system in the rat. The finding of dissociation between approach motivation and baseline USVs as behavioural predictors may indicate that models of dopamine function such as incentive salience are not fully adequate to

account for 50 kHz USV emission. This is in line with prior research involving food-reward related conditioning that found attribution of incentive salience could occur independently of emission of USVs (Brenes & Schwarting, 2014). However, the emission of USVs may still be indicative of incentive salience, which would account for the positive relationship found between rates of emission to reward-cue and cue-related anticipatory activity (Brenes & Schwarting, 2015).

As discussed in chapter 2 an unfortunate limitation encountered was the inability to effectively incorporate sucrose preference into predictive models of 50 kHz USV behavioural response to AMPH. Had individual measures of sucrose preference correlated with other predictors it may have allowed for aspects of hedonia or 'liking' to be conceptually extracted from the USV and approach motivation components. Such a framework may have provided evidence to determine if 50 kHz USVs and the predisposition to call offer a link between the liking and wanting aspects that dopamine function is typically dichotomized into. Further, this may have elucidated a utility of 50 kHz USV measurement in bridging anticipatory paradigms (where reward associated behaviours are measured in anticipation of the stimulus) with consummatory paradigms (where reward associated behaviours are measured upon reception/interaction with stimulus).

The literature is replete with many examples of 50 kHz USV emission being found in both anticipatory (Knutson et al., 1998; Burgdorf et al., 2000; Buck, Malavar, et al., 2014) and consummatory situations (Burgdorf et al., 2008; Kisko, Wöhr, et al., 2015). The results found for the social conditions in chapter 3 may provide additional support for the notion that emission of 50 kHz USVs may bridge these anticipatory and

consummatory aspects. For these social conditions the rats emitted 50 kHz USVs recorded in the absence of the conspecific. The USV emission in such a case may reflect an internal state of residual expectancy developed in the preceding 5 min (when conspecific was present). These findings may then suggest that even in a context of putatively frustrated consummation their expression serves as a behavioural measure of internal organismal states.

Frequency modulation in 50 kHz USVs as an index of positive emotional arousal

It has been postulated that the frequency modulation associated with some 50 kHz USVs may be the aspect of emission most reflective of an underlying positive emotional state (Burgdorf et al., 2011). This idea is consistent with the prevalent findings of FM subtypes occurring in high proportion in situations with high positive emotional arousal such as playful interaction in juveniles (Burgdorf et al., 2008; Himmler, Kisko, Euston, Kolb, & Pellis, 2014), or reward seeking behaviour related to dopaminergic psychostimulant administration (Mahler et al., 2013). There have been reports of psychostimulant-induced sensitization of FM 50 kHz USVs specifically within repeat-administration protocols (Ahrens et al., 2009; Taracha et al., 2012). Some FM 50 kHz USVs, such as the trill subtype, appear particularly associated with the effects of catecholamine agonists like AMPH (Wright et al., 2010; Simola et al., 2012; Simola, Frau, Plumitallo, & Morelli, 2014). Moreover, FM 50 kHz subtypes appear especially vulnerable to disruptions of the mesolimbic dopamine system while flat subtypes are often resilient (Burgdorf et al., 2007; Ciucci et al., 2009).

The degree of frequency modulation measured among emitted 50 kHz USVs may serve to quantitatively index the magnitude of the underlying positive emotional arousal.

My research findings from chapter 3 and 4 provide empirical observations consistent with this notion. In chapter 3 it was found that the most powerful 50 kHz USV inducing stimuli (the social conditions and in particular the female conspecific condition) also preferentially increased FM 50 kHz USV subtypes. In contrast, the much weaker USV inducing stimuli (the non-social consumables) appeared to preferentially increase flat 50 kHz USV subtypes.

It must be explicitly noted that the findings from chapter 3 do not allow for determination of any exact cause of the observed subtype alteration across conditions. As mentioned in the chapter-specific discussion section a limitation was that the experimental procedure did not control for motivation differences across the stimuli. While it is possible that the female stimulus increased FM 50 kHz USVs when compared with the other stimuli as a function of differences in appetitive motivation the data to not allow such a determination. It is alternatively conceivable that the social and non-social behavioural contexts elicited subtype-specific USV emission in accordance with general notions of communication, the nuances of which may have been lost in the gross coding scheme utilized in chapter 3. Relatively high-resolution 50 kHz USV subtype coding schemes have found evidence of behaviour-specific associations in social contexts (Burke, Kisko, Swiftwolfe, Pellis, & Euston, 2017; Burke, Kisko, Euston, & Pellis, 2018). Moreover, there is evidence that various FM subtypes may differentially relate to reward processing (Garcia, McCowan, & Cain, 2015). Regardless of the specific cause of such differences in subtypes, however, the postulation that frequency modulation is a characteristic of 50 kHz USV emission most highly associated with positive emotional arousal appears consistent with such literature and my empirical findings.

The apparent capacity of dopaminergic psychostimulant drugs to preferentially induce FM 50 kHz USVs has provided support for the inference that such a phenomenon is reflective of the signaling actions of norepinephrine in the brain in addition to dopamine (Wright et al., 2010; Rippberger et al., 2015). Indeed, drugs such as AMPH profoundly alter both catecholaminergic systems (Sulzer et al., 2005). Additionally, there is evidence from administration of more selective adrenergic drugs, which indicates a dependence of frequency modulation of 50 kHz USVs on norepinephrine signaling (Wright, Dobosiewicz, & Clarke, 2012). The systemic administration of propranolol (a β1/β2 antagonist), for instance, was found capable of selectively blocking AMPH-induced increases in certain FM 50 kHz USVs while promoting flat subtypes. Thus, the total call-rate increase of AMPH was maintained following propranolol pre-treatment, but the type of calls typically promoted by AMPH (FM) were selectively reduced (Wright et al., 2012). These findings raise the interesting question of whether the relation of 50 kHz call rate and frequency modulation often seen in the psychostimulant literature is closely associated with dopamine signaling.

As previously mentioned, my findings from chapter 3 were that non-psychostimulant-induced alterations in frequency modulation could be blocked by an antagonist of dopamine receptors. This is consistent with the proposed close association between FM call subtypes and dopamine signaling. However, more related to the psychostimulant literature specifically is the finding of a positive correlation between frequency modulation and call rate following local application of dopamine into the NAc shell from chapter 4. This indicates that an increased concentration of dopamine within the NAc itself is capable of producing such a phenomenon. This casts doubt on the notion

that the relation of frequency modulation and 50 kHz call rate observed following psychostimulant induction is an effect of the broad-spectrum of action such drugs exert (both centrally and peripherally).

Delineation of functional subcomponents for positive emotional expression via 50 kHz USV emission and ergometric activity

As outlined in the general introduction the neuroanatomy of the ascending mesolimbic dopamine projections appears consistent with the view of functional segregation between limbic and motor circuits (Ikemoto, 2007). The general conceptual division of the striatum into ventromedial and dorsolateral components (Voorn et al., 2004) does not preclude information flow between limbic-related and motor-related circuits (Mogenson et al., 1980; Floresco, 2015). The capacity for translation of emotionally relevant information into coordinated motor-action patterns is a conceptual necessity for frameworks of emotional states. The manifestation of increased locomotor and motor activity in rats is well established as a behavioural metric of such conceived emotional states (Kelley, 1993; Blakemore & Vuilleumier, 2017). The metric of locomotor activity has thus been central to behavioural-pharmacology investigations of the role of dopamine in motivated and emotional states of arousal (Deminiere, Piazza, Le Moal, & Simon, 1989; Hooks et al., 1991).

Recent investigations have provided evidence that psychostimulant-induced behavioural sensitization differentially affects locomotor and 50 kHz USV measurements in adult rats (Taracha et al., 2014; Garcia & Cain, 2016). Thus, while AMPH administration may increase manifestation of both locomotor activity and 50 kHz USVs the behavioural responses are separable. Locomotor activity has been strongly associated

with exploratory behaviour (Kelley, 1993), and thus arguably, aspects of approach motivation. It is conceivable that the separation between locomotor activity and 50 kHz USV behaviour could be indicative of the same dissociation probed in chapter 2. However, in chapter 5 I found evidence that this separation in behavioural response to AMPH between USV and motor activity could be extended beyond locomotion specifically to two forms of general ergometric activity. My findings in chapter 5 indicated that both large and small bodily motions captured by an ergometric stage were dissociable from 50 kHz USV emission with respect to individual differences in sensitization. This form of motor arousal appears less agreeable to an approach motivation interpretation and may effectively indicate an earlier stage of limbic-to-motor information progression. These findings thus appear consistent with a possible functional division in the role that dopamine plays in the ventromedial-dorsolateral striatal system. It may be that these non-overlapping behavioural responses to psychostimulants inform upon different aspects of the underlying brain network architecture in regard to translation of limbic-related information to motor-action.

In chapter 5, however, I failed to find evidence in support of medial prefrontal (PL and IL cortices), medial striatum, and BLA directly related to manifest 50 kHz USV behaviour after AMPH. There was no significant first-order correlation between the inducible transcription factor Zif in any of the regions of interest and recorded behaviour. Exploratory analyses I conducted in chapter 5 did provide speculative evidence of associations in immunostaining between prefrontal and striatal regions within a subgroup of the sample characterized by a high duration of 50 kHz calling. This analysis tentatively suggests that the relation of PL cortex and NAcSh and the IL and DMS may be

associated with intense 50 kHz USV emission following AMPH. While my research findings from chapter 5 may only be capable of indicating this as a direction of future research, the finding of a possible involvement of the prefrontal cortex in expressions of positive emotional arousal is not surprising. Indeed, the prefrontal region is a central terminal region in the subset of ascending dopamine projections most likely to be associated with establishing such positive emotional arousal (Ikemoto, 2007; Lammel et al., 2008; Beier et al., 2015). Intriguingly, such a prefrontal projecting subcomponent would align with Maclean's postulation that the limbic brain and prefrontal neocortex represent an evolved mechanism to fulfill a variety of 'family' oriented behavioural roles (MacLean, 1955, 1985).

Conclusion

The utility of rat USVs as a behavioural measure of emotion is increasingly being recognized in research investigating drug and gambling addiction, animal models in medicine, and the well-being of animals in facilities (Wöhr & Schwarting, 2013; Cloutier, Wahl, Panksepp, & Newberry, 2015; Barker et al., 2015). Thus, there is an ever-greater obligation for clarity and understanding in the nature of how these vocalizations express the internal states of the organism. My findings from chapters 2 to 5 provide some increased measure of characterization of how the 50 kHz USVs of the rat express positive emotional arousal.

My findings from chapter 2 indicate that the 50 kHz USVs induced by acute AMPH may reflect more than an individual rats' approach motivation characteristic. These findings suggest possible non-overlap between models of motivation and the emotional arousal putatively indexed by 50 kHz USVs. In chapter 3, I found evidence

that the 50 kHz USVs of the adult rat could be non-pharmacologically induced and modulated in behaviourally relevant scenarios. Moreover, regardless of the behavioural context, all 50 kHz calling could be blocked by systemic administration of a dopamine antagonist (HAL). Thus, chapter 2 and 3 helped to characterize 50 kHz USVs at a behavioural level both within-subjects and between. In chapter 4, I found evidence that dopamine acting locally in the NAcSh is capable of inducing 50 kHz USV emission. The character of calling was also found to be comparable to AMPH in that there was a linear increase in frequency modulation with call rate. Thus chapter 4 and the dopamine antagonism in chapter 3 serve to characterize 50 kHz USVs at a pharmacological level. In chapter 5, I found evidence of separation between 50 kHz USVs and ergometric activity in response to a two-injection protocol of sensitization using AMPH. Additionally, there was some indication from correlated immunostained brain regions within a subgroup with intense USV emission that the prefrontal cortex is involved in the behavioural responses observed following this sensitized AMPH. This serves to characterize 50 kHz USVs at an immunohistochemical level.

In aggregate, my empirical findings are consistent with the existence of a putative subcomponent of the ascending mesolimbic dopamine system responsible for positive emotional arousal reflected by 50 kHz USVs in the rat. Such a system may operate to produce 50 kHz USV emission in a manner not directly reflected by measures of approach motivation. The overt manifestation of the activity of such a system is subject to behaviourally-relevant modulation, potentially indexed in 50 kHz USVs as frequency modulation. Such a frequency modulation index is closely associated with the function of dopamine in the ventral striatum and may be reproduced by its direct application.

Additionally, the emission of 50 kHz USVs reflects activity of underlying brain systems in a manner distinct from gross measures of bodily activity. The extent of operation of such a system may also, and more speculatively, be beyond the NAc and is likely embedded in complex prefrontal-striatal circuitry.

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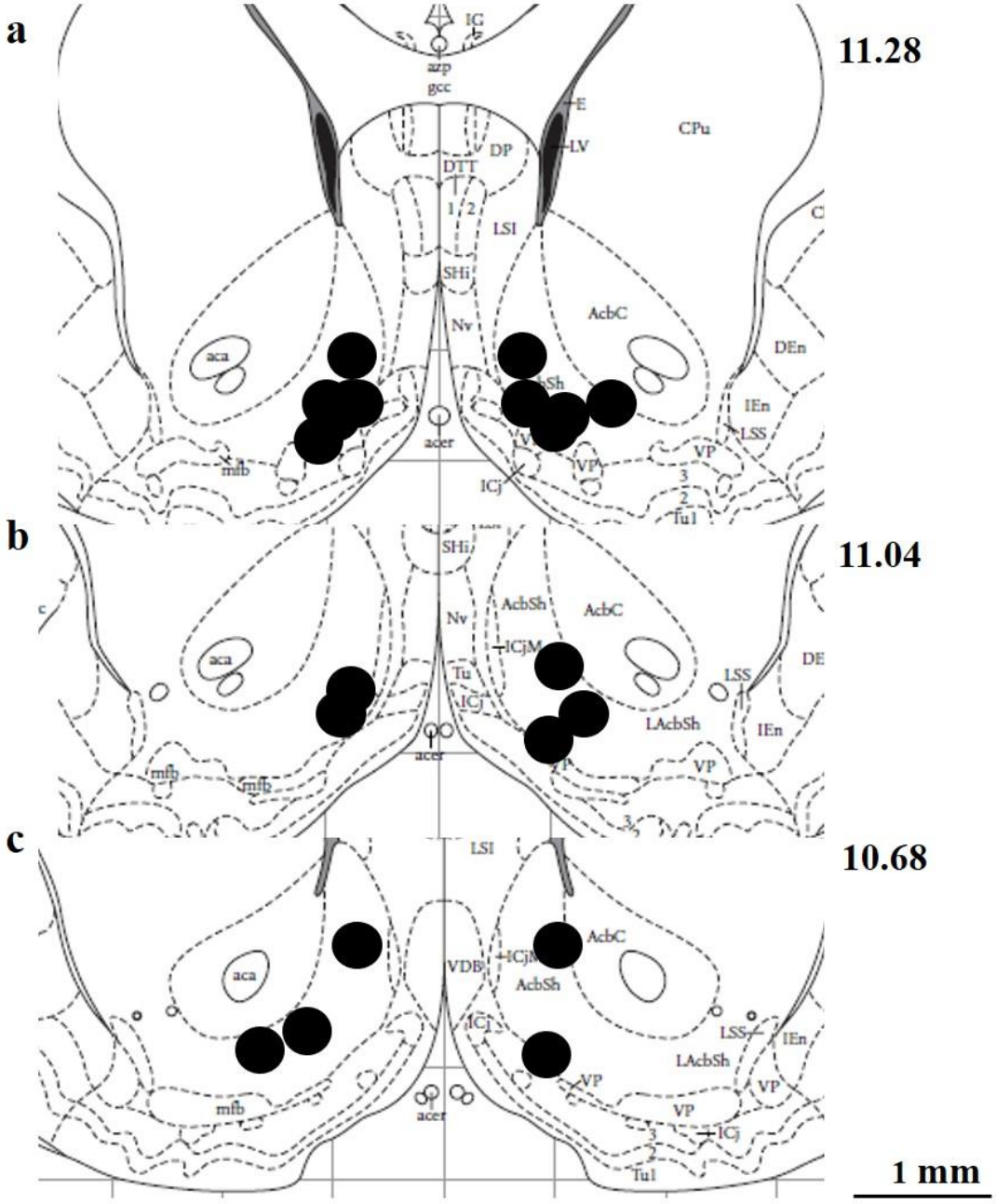
Appendix A



*Localization of valid injection sites utilized in all experimental groups on the coronal sections of the rat brain according to the stereotaxic atlas by Paxinos and Watson (1986). Panels **a**, **b**, and **c** represent movement along the anterior-posterior axis from 11.28 mm to 10.68 mm from the interaural zero plane (numbers on the top right corner of each panel). For sake of clearness any directly overlapping sites are not shown. Bar at bottom right corner represents 1 mm for scale. 11 valid subjects localized in left hemisphere, 14 localized in right hemisphere. Abbreviations: aca – anterior commissure, anterior part; AcbC – accumbens nucleus, core; AcbSh – accumbens nucleus, shell; CPu*

– caudate putamen (striatum); LacbSh – lateral accumbens shell; LSI – lateral septal nucleus, intermediate part; mfb – medial forebrain bundle; VP – ventral pallidum.

Appendix B

Brock

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: March 11, 2015

Dear Dr. Brudzynski and Mr. Mulvihill,

Your "Animal Use Protocol (AUP)" entitled:

Behavioural and neurophysiological differences between Long Evans rats that emit a high versus low number of50 kHz USVs.

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 15 - 03 - 03**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 46 Long Evans rats from AUP 14-11-01 (CMc)

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix C

Animal Care Committee (ACC)
Chair –Fiona Hunter, PhD 905.688.5550 ext 3394
ACC Veterinarian – Alistair Ker, DVM. 905.227.7644
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: April 24, 2014

Dear Dr. Brudzynski and Mr. Silkstone,

Your "Animal Use Protocol (AUP)" entitled:

Mapping brain structures along the ascending mesolimbic dopamine system that can initiate 50-kHz ultrasonic vocalizations in the rat.

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 14 - 04 - 04**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 120 Long evans male rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Fiona Hunter, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.

Appendix D

Brock

Animal Care Committee (ACC)
Chair –Wendy Ward, PhD 905.688.5550 ext 3024
Clinical Veterinarians – Dr. Alistair Ker and Dr. Susan Warren
Animal Care Committee Coordinator – Dayle Carlson, RMLAT 905.688.5550 ext 5820

Date: August 19, 2015

Dear Dr. Brudzynski and Mr. Mulvihill

Your "Animal Use Protocol (AUP)" entitled:

Mapping of neural substrates associated with 50 kHz calling in behavioural contexts with immediate early genes in the Long Evans rat.

has been approved by the Animal Care Committee.

This approval expires in one year on the last day of the month.
The number for this project is **AUP # 15 - 08 - 01**.
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 40 male and 2 female Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Wendy Ward

Wendy Ward, Chair of ACC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY AND IS SUBJECT TO POST APPROVAL MONITORING.

ALL UNEXPECTED MORTALITIES MUST BE REPORTED TO ANIMAL CARE SERVICES STAFF IMMEDIATELY.